ABSTRACT

Endothelium-dependent relaxations are impaired in carotid artery of apolipoprotein E-deficient (apoE<sup>−/−</sup>) mice. This impairment seems to be due to increased formation of superoxide anions and inactivation of endothelial nitric oxide (NO). In the present study, we tested hypothesis that chronic treatment with vitamin C may prevent endothelial dysfunction by increasing release of NO from endothelial cells. C57BL/6 and apoE<sup>−/−</sup> mice were treated for 26 weeks with Western-type fat diet with and without 1% vitamin C. Vasomotor function of isolated carotid arteries was studied by video dimension analyzer. Expression of endothelial NO synthase (eNOS) and platelet-endothelial cell adhesion molecule-1 (PECAM-1) protein were evaluated by Western blotting. Levels of cGMP and cAMP were measured by radioimmunoassay. In apoE<sup>−/−</sup> mice, vitamin C significantly augmented relaxations to acetylcholine (10<sup>−9</sup>–10<sup>−5</sup> mol/l), but did not affect relaxations to NO donor diethylammonium-(Z)-1-(N,N-diethylamino) diazen-1-1,2-diolate (DEA-NONOate; 10<sup>−9</sup>–10<sup>−5</sup> mol/l). In contrast, vitamin C reduced relaxations to acetylcholine and DEA-NONOate in C57BL/6 mice. Interestingly, vitamin C significantly increased basal cGMP levels in C57BL/6 mice but did not affect cGMP formation in apoE<sup>−/−</sup> mice. Vitamin treatment did not affect expression of eNOS protein, whereas elevated expression of PECAM-1 protein in apoE<sup>−/−</sup> mice was returned to normal level. Our findings demonstrate that chronic treatment with vitamin C prevents endothelial dysfunction of carotid artery induced by hypercholesterolemia. This effect seems to be mediated by preservation of NO bioavailability in endothelial cells.

Hypercholesterolemia is a major risk factor for development of cardiovascular disease. Dysfunction of vascular endothelial cells due to impaired production and/or biological activity of NO seems to play a key role in initiation and development of atherosclerosis (Zeier et al., 1991; Cai and Harrison, 2000). Adhesion molecules, including PECAM-1, contribute to pathogenesis of atherosclerosis (O'Brien et al., 1996; Poston and Johnson-Tidey, 1996). The exact molecular mechanisms underlying endothelial dysfunction in arteries exposed to hypercholesterolemia are not completely understood. In a murine model of hypercholesterolemia and atherosclerosis, apoE<sup>−/−</sup> mice (Plump et al., 1992; Zhang et al., 1992), we demonstrated that endothelial function is impaired in carotid artery even before any morphological changes could be detected in arterial wall (d'Uscio et al., 2001). This impairment was due to increased formation of superoxide anion, a chemical antagonist of NO (d'Uscio et al., 2001).

In our previous study, we demonstrated that long-term treatment with vitamin C has a beneficial effect on endothelial function of apoE<sup>−/−</sup> aorta (d'Uscio et al., 2003). However, it is well established that pharmacology of cerebrovascular tree is different from pharmacology of peripheral circulation (Rang et al., 2001). Therefore, the rationale for the present study was based on the fact that in vivo effect of chronic vitamin C treatment on endothelial function of carotid arteries has not been studied. Furthermore, understanding of mechanisms responsible for beneficial effects of vitamin C on vascular function in vivo is incomplete. We hypothesized that chronic treatment with an antioxidant, vitamin C, may protect endothelial function of carotid arteries by preserving normal bioavailability of nitric oxide. If correct, this hypothe-
esis may also help to explain the mechanisms underlying decreased risk of stroke in humans with high plasma concentra-
tion of vitamin C (Simon et al., 1998).

Materials and Methods

Animal Groups. Male C57BL/6J (wild-type) mice and homozy-
gous apoE-deficient mice (C57BL/6J-ApoE−/−) were obtained at the age of 4 to 5 weeks from The Jackson Laboratory (Bar Harbor, ME). Housing facilities and all experimental protocols were approved by the Institutional Animal Care and Use Committee of Mayo Clinic (Rochester, MN). C57BL/6J mice and apoE-deficient mice were fed for 26 weeks a lipid-rich Western-type diet (0.15% cholesterol and 42% milk fat by weight, TD88137; Harlan Teklad, Madison, WI) without or with Vitamin C (1%/kg diet) (Plump et al., 1992).

Blood Sample and Body Weight. Blood weight was measured with triple beam balance (Ohaus, Florham Park, NJ). Blood samples were obtained through puncture of the right ventricle. The blood was immediately transferred to a tube containing heparin (1000 U) and centrifuged at 4°C for 10 min. Plasma was separated immediately at 4°C and kept at −80°C until assayed. Plasma total cholesterol was determined using a colorimetric-based assay on a Cobas Mira system. Vitamin C was stabilized with 10% meta-phosphoric acid by removing protein and analyzed by UV absorbance after elution from a reversed-phase high-performance liquid chromatography with phosphate buffer (pH 2).

Analysis of Vascular Reactivity with Arteriography. Experi-
ments were performed on 7-mm-long carotid rings from mice that had been anesthetized with pentobarbital (60 mg/kg i.p.) as detailed in our previous study (d’Uscio et al., 2003). Carotid arteries were carefully removed and placed immediately into cold (4°C) modified Krebs-Ringer bicarbonate solution (118.6 mmol/l NaCl, 4.7 mmol/l KCl, 2.5 mmol/l CaCl2, 1.2 mmol/l MgSO4, 1.2 mmol/l KH2PO4, 25.1 mmol/l NaHCO3, 0.026 mmol/l calcium-ethylenediamine-tetraacetic acid, and 10.1 mmol/l glucose). Carotid arteries were dissected free from connective tissue in cold Krebs’ solution and transferred to an arteriograph (Living Systems Instrumentation, Burlington, VT). At the beginning of each experiment, vessels were equilibrated for 45 min at 50 mm Hg and wall thickness and diameter were measured. Vessels were contracted with thromboxane analog 9,11-dideoxy-
min at 50 mm Hg and wall thickness and diameter were measured.

Drugs and Chemical Agents. Acetylcholine hydrochloride and
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3-isobutyl-1-methylxanthine (IBMX) were from Sigma-Aldrich (St. Louis, MO). DEA-NONOate and U-46619 were from Cayman Chemical (Ann Arbor, MI). DEA-NONOate was prepared as stock solutions in 1.5 mol/l Tris buffer, pH 8.8. U46619 was dissolved in 1 part of 100% ethanol and then diluted with 9 parts of water. The remaining drugs were dissolved in distilled water. All drugs except IBMX were then diluted in Krebs’ solution and concentrations are expressed as final molar concentration (moles per liter).

Measurement of the Level of cGMP and cAMP in the Carotid Artery. A radiodimmunoassay technique was used to determine the levels of cGMP and cAMP, as reported previously (d’Uscio et al., 2001). Artery was initially incubated in minimal essential media with 10% horse serum and 1% penicillin-strepto-
mucin (Invitrogen, Carlsbad, CA) in a 5% CO2 incubator at 37°C for 30 min. After this 30-min period, tissue was incubated another 30 min in IBMX (10−4 mol/l) to inhibit the degradation of cyclic nucle-

Western Blot Analysis. Western blot was done as reported pre-
viously (d’Uscio et al., 2001). Briefly, after collection and removal of connective tissue, carotid arteries were homogenized on ice in lysis buffer (pH 7.5) containing 50 mM Tris-HCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% SDS, 0.1% deoxycholate, 1% IGEPAL, and a 100-fold dilution of a mamalian protease inhibitor cocktail (all from Sigma-Aldrich). Equal amounts of protein (30 μg/lane) were separated by 7.5% SDS-PAGE and transferred to nitrocellulose membrane (Am-
ersham Biosciences, Inc.). For eNOS protein analysis, monocular anti-eNOS (1:100; Transduction Laboratories, San Diego, CA) was used. For PECAM-1 protein analysis, polyclonal anti-PECAM-1 (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used. Actin (1:50,000; Sigma-Aldrich) was used as internal control. Bands were visualized by enhanced chemiluminescence using a commer-
cially available kit (Amersham Biosciences, Inc.). Densitometry was carried out using NIH Image (Scion-Image; Scion Corporation, Fred-
river, MD), and the results were expressed in relative densitometry compared with actin.

Statistical Analysis. Results are expressed as mean ± S.E.M., for n animals used in each experimental protocol. One-way analysis of variance with multiple comparisons adjustment (Dunnett’s method) determined the statistical significance of differences between the vitamin C, total cholesterol, cGMP, cAMP, and optical intensity values in the different experimental groups. Repeated measures analysis of variance with multiple comparisons adjustment (Bonferroni’s method) determined the statistical significance of differ-
ces between contraction and relaxation levels in the different experimental groups. A value of P < 0.05 was considered statistically significant.

Results

Body Weight and Morphological Data. The body weights were 48.6 ± 0.7, 47.8 ± 0.5, 36.1 ± 1.5,8 and 35.7 ± 1.7,6 g for C57BL/6J mice, C57BL/6J mice treated with vitamin C, apoE−/− mice, and apoE−/− mice treated with vitamin C, respectively (*P < 0.05 versus C57BL/6J mice and C57BL/6J mice treated with vitamin C; n = 16–17). Diameters of the carotid arteries were 432.6 ± 8.38, 419.2 ± 4.68, 448.2 ± 9.02, and 423.9 ± 9.40 μm in C57BL/6J mice, C57BL/6J mice treated with vitamin C, apoE−/− mice, and apoE−/− mice treated with vitamin C, respectively (P = N.S.; n = 15–22). Wall thicknesses of the carotid arteries were 48.0 ± 1.65, 49.9 ± 1.52, 54.3 ± 1.72,8 and 48.4 ± 1.53 μm in C57BL/6J mice, C57BL/6J mice treated with vitamin C, apoE−/− mice, and apoE−/− mice treated with vitamin C, respectively (*P < 0.05 versus C57BL/6J mice, C57BL/6J mice treated with vitamin C, and apoE−/− mice treated with vitamin C; n = 15–22).

Plasma Vitamin C and Total Cholesterol. Plasma vita-
in C levels were significantly lower in apoE−/− mice compared with C57BL/6J mice (Fig. 1A). Chronic vitamin C treatment increased 2- to 3-fold in apoE−/− mice and apoE−/− mice treated with vitamin C compared with untreated animals (Fig. 1A). Plasma total cholesterol levels were signifi-
cantly higher in apoE−/− mice and apoE−/− mice treated with vitamin C compared with C57BL/6J mice and C57BL/6J mice treated with vitamin C (Fig. 1B). Chronic vitamin C treat-
ment had no effect on the cholesterol level in apoE−/− mice.

Vascular Reactivity. Concentration-response curves to U46619 were not significantly different among four groups of mice (Fig. 2). During submaximal contractions to U46619, endothelium-dependent relaxations to acetylcholine were significantly impaired in the carotid artery of C57BL/6J mice
treated with vitamin C (Fig. 3A). Endothelium-independent relaxations to the NO donor DEA-NONOate were also significantly impaired in the carotid artery of C57BL/6J mice treated with vitamin C (Fig. 3B). In contrast, endothelium-dependent relaxations to acetylcholine were normalized in the carotid artery of apoE−/− mice treated with vitamin C (Fig. 4A). Endothelium-independent relaxations to the NO donor DEA-NONOate were not different between apoE−/− mice treated with vitamin C and apoE−/− mice treated with vitamin C (Fig. 4B).

cGMP and cAMP Levels. Basal cGMP levels were significantly increased in carotid arteries of C57BL/6J mice treated with vitamin C compared with C57BL/6J, apoE−/−/− mice, and apoE−/−/− mice treated with vitamin C (Fig. 5A). In contrast, basal cAMP levels were not different among the four groups (Fig. 5B).

eNOS Protein Expression. Western blot analysis detected similar eNOS protein expression in common carotid arteries of C57BL/6J, C57BL/6J treated with vitamin C, apoE−/−/− mice, and apoE−/−/− mice treated with vitamin C (Fig. 6).

PECAM-1 Protein Expression. PECAM-1 expression was increased in apoE−/− mice compared with C57BL/6J. Vitamin C treatment normalized the elevated expression of PECAM-1 protein in apoE−/− mice (Fig. 7).

Discussion

This is the first study to examine the effect of chronic vitamin C treatment on vascular function of mouse carotid artery. In C57BL/6J mice, vitamin C significantly increased...
basal production of cGMP, most likely reflecting increased formation and release of NO. Indeed, chronic vitamin C treatment increases nitric-oxide synthase enzymatic activity in aortas of C57BL/6J mice (d’Uscio et al., 2003). Consistent with previous reports, increased local concentration of vascular NO caused reduced smooth muscle reactivity to both endogenous NO released by acetylcholine or exogenous NO generated by DEA-NONOate (Molina et al., 1987; Ohashi et al., 1998; d’Uscio et al., 2003). However, the most important finding of the present study is that chronic treatment with vitamin C has beneficial effect on endothelial function in hypercholesterolemic apoE<sup>−/−</sup> mice carotid artery. These results are in agreement with our previous report demonstrating an important role of reactive oxygen species in pathogenesis of endothelial dysfunction in apoE<sup>−/−</sup> carotid artery (d’Uscio et al., 2001). Unlike humans who depend on dietary intake to maintain normal circulating levels of vitamin C, mice are capable of synthesizing vitamin C (Bhattacharjee et al., 1985). Interestingly, mice and humans have comparable levels of plasma vitamin C (0.6–2.0 mg/dl; Levine et al., 1999). In apoE<sup>−/−</sup> mice, we observed that plasma levels of vitamin C were significantly decreased compared with levels in C57BL/6J animals. Although the reason for this decrease is not apparent, it is most likely result of increased vitamin C catabolism due to hypercholesterolemia-induced oxidative stress (Muldoon et al., 1996). Low plasma vitamin C is associated with increased risk of stroke (Kurl et al., 2002). As expected, high cholesterol levels were detected in plasma of apoE<sup>−/−</sup> mice and they were not affected by vitamin C treatment.

Vasodilatation in response to acetylcholine and DEA-NONOate was studied in arteries contracted with a thromboxane A<sub>2</sub> analog, U46619. We did not detect any difference...
PECAM-1 is a 130-kDa member of immunoglobulin superfamily that is expressed on the surface of circulating platelets, monocytes, neutrophils, selected T-cell subsets, and vascular endothelial cells (Davies et al., 1993). PECAM-1 participates in the adhesion cascade leading to extravasation of leukocytes to sites of inflammation (Vaporiyan et al., 1993). Pretreating monocytes or neutrophils with antibodies specific for PECAM-1 inhibits their emigration across vascular endothelial cells. We demonstrated that elevated expression of PECAM-1 in apoE \( ^{-/-} \) mice was returned to normal level by vitamin C treatment. This may be an important mechanism underlying antiatherosclerotic effect of vitamin C (Lehr et al., 1994, 1995; Weber et al., 1996). Reduction of the leukocytes migration into the vascular wall can reduce local concentrations of superoxide anion (Weber et al., 1996) and contribute to restoration of NO biological activity.

The results of the present study demonstrate that chronic vitamin C treatment prevents endothelial dysfunction in carotid artery of apoE \( ^{-/-} \) mice. This effect seems to be mediated by increased bioavailability of endothelial NO. We speculate that in humans dietary intake (or supplementation) of vitamin C could be an important factor in preservation of normal endothelial function of carotid artery and prevention of stroke.

The most striking finding of the present study is that vitamin C significantly improved endothelium-dependent relaxations to acetylcholine in carotid artery of apoE \( ^{-/-} \) mice. The effect was most likely due to increased bioavailability of NO in endothelium because vitamin C did not affect relaxation of smooth muscle cells to DEA-NONOate. The exact mechanism underlying beneficial effect of vitamin C is not clear. Despite the fact that eNOS protein expression is not affected by vitamin C (Heller et al., 1999; Baker et al., 2001), we may have underestimated eNOS expression in ApoE \( ^{-/-} \) mice due to increased carotid artery wall thickness. Therefore, up-regulation of eNOS expression in ApoE \( ^{-/-} \) carotid arteries cannot be completely ruled out. Several other mechanisms may account for the observed improvement in endothelial function, including scavenging of superoxide anions (Jackson et al., 1998) and stabilization of eNOS cofactor tetrahydrobiopterin (Huang et al., 2000; Baker et al., 2001; Heller et al., 2001; d’Uscio et al., 2003). Our previous study provided evidence that long-term vitamin C treatment may protect tetrahydrobiopterin from oxidation in aortas of apoE \( ^{-/-} \) carotid arteries (d’Uscio et al., 2003). In the present study, we did not measure tetrahydrobiopterin levels because of the technical difficulties caused by very small size of the mouse carotid arteries.

The results of the present study indicated that wall thickness is increased in apoE \( ^{-/-} \) mice. Vitamin C treatment prevented this change in vascular structure. The exact mechanism underlying increased wall thickness of apoE \( ^{-/-} \) carotid arteries and beneficial effect of vitamin C is unclear. However, our findings are consistent with the reported inverse relationship between vitamin C intake and carotid artery wall thickness in population included in Atherosclerotic Risk in Communities Study (Kritchevsky et al., 1995). Increase in carotid artery thickness is also consistent with reported increase in arterial blood pressure of apoE \( ^{-/-} \) (Yang et al., 1999; Buzello et al., 2003).

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