Protective Effect of Chronic Vitamin C Treatment on Endothelial Function of Apolipoprotein E-Deficient Mouse Carotid Artery

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ABSTRACT

Endothelium-dependent relaxations are impaired in carotid artery of apolipoprotein E-deficient (apoE−/−) 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 the hypothesis that chronic treatment with vitamin C may prevent endothelial dysfunction by increasing release of NO from endothelial cells. C57BL/6 and apoE−/− 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−/− mice, vitamin C significantly augmented relaxations to acetylcholine (10−9–10−5 mol/l), but did not affect relaxations to NO donor diethylammonium-(Z)-1-(N,N-diethylamino) diazen-1-1,2-diolate (DEA-NONOate; 10−9–10−5 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−/− mice. Vitamin treatment did not affect expression of eNOS protein, whereas elevated expression of PECAM-1 protein in apoE−/− 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 (Zeiger 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-Tiday, 1996). The exact molecular mechanisms underlying endothelial dysfunction in arteries exposed to hypercholesterolemia have not been completely understood. In a murine model of hypercholesterolemia and atherosclerosis, apoE−/− 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−/− 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 concentration of vitamin C (Simon et al., 1998).

Materials and Methods

Animal Groups. Male C57BL/6J (wild-type) mice and homozygous 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. Body 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 was obtained through puncture of the right ventricle. The blood was without or with Vitamin C (1%/kg diet) (Plump et al., 1992).

Analysis of Vascular Reactivity with Arteriography. Experiments 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-

Plasma vitamin C and total cholesterol. Plasma vitamin C levels were significantly lower in apoE−/− mice compared with untreated animals (Fig. 1A). Plasma vitamin C treatment increased 2- to 3-fold in apoE−/− mice treated with vitamin C, respectively (*P < 0.05 versus C57BL/6J 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 = 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, 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 vitamin 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−/− and C57BL/6J mice treated with vitamin C compared with untreated animals (Fig. 1A). Plasmap total cholesterol levels were significantly 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 treatment 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. 1C). 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<sup>−/−</sup>/H<sup>11002</sup>/H<sup>11002</sup>/H<sup>11002</sup> mice treated with vitamin C (Fig. 4A). Endothelium-independent relaxations to the NO donor DEA-NONOate were not different between apoE<sup>−/−</sup>/H<sup>11002</sup>/H<sup>11002</sup>/H<sup>11002</sup> mice and apoE<sup>−/−</sup>/H<sup>11002</sup>/H<sup>11002</sup>/H<sup>11002</sup> 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<sup>−/−</sup>/H<sup>11002</sup>/H<sup>11002</sup>/H<sup>11002</sup> mice, and apoE<sup>−/−</sup>/H<sup>11002</sup>/H<sup>11002</sup>/H<sup>11002</sup> 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<sup>−/−</sup>/H<sup>11002</sup>/H<sup>11002</sup>/H<sup>11002</sup> mice, and apoE<sup>−/−</sup>/H<sup>11002</sup>/H<sup>11002</sup>/H<sup>11002</sup> mice treated with vitamin C (Fig. 6).  

**PECAM-1 Protein Expression.** PECAM-1 expression was increased in apoE<sup>−/−</sup>/H<sup>11002</sup>/H<sup>11002</sup>/H<sup>11002</sup> mice compared with C57BL/6J. Vitamin C treatment normalized the elevated expression of PECAM-1 protein in apoE<sup>−/−</sup>/H<sup>11002</sup>/H<sup>11002</sup>/H<sup>11002</sup> 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−/− 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−/− 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−/− 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−/− 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 A2 analog, U46619. We did not detect any difference in relaxation to acetylcholine or DEA-NONOate in apoE−/− mice treated with vitamin C compared with apoE−/− mice treated without vitamin C (P < 0.05; analysis of variance (ANOVA) + Bonferroni’s; n = 6). B, no differences were found between apoE−/− mice and apoE−/− mice treated with vitamin C (P = N.S.; ANOVA + Bonferroni’s; n = 6–7) (ApoE, apoE−/− mice; Vit C, vitamin C).
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 (Vaporciyan 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<sup>−/−</sup> 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). The reduction of the leukocytes migration into the vascular wall can reduce local mechanism underlying antiatherosclerotic effect of vitamin C 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<sup>−/−</sup> 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.
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References


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