Ascorbic acid enhances NO bioactivity in patients with vascular disease through unclear mechanism(s). We investigated the role of intracellular ascorbic acid in endothelium-derived NO bioactivity. Incubation of porcine aortic endothelial cells (PAECs) with ascorbic acid produced time- and dose-dependent intracellular ascorbic acid accumulation that enhanced NO bioactivity by 70% measured as A23187-induced cGMP accumulation. This effect was due to enhanced NO production because ascorbate stimulated both PAEC nitrogen oxide (NO₂⁻ + NO) production and L-arginine to L-citrulline conversion by 59% and 72%, respectively, without altering the cGMP response to authentic NO. Ascorbic acid also stimulated the catalytic activity of eNOS derived from either PAEC membrane fractions or baculovirus-infected Sf9 cells. Ascorbic acid enhanced bovine eNOS V₅₀ by ~50% without altering the Kₘ for L-arginine. The effect of ascorbate was tetrahydrobipterin (BH₄)-dependent, because ascorbate was ineffective with BH₄ concentrations >10 μM or in PAECs treated with sepiapterin to increase intracellular BH₄. The effect of ascorbic acid was also specific because A23187-stimulated cGMP accumulation in PAECs was insensitive to intracellular glutathione manipulation and only ascorbic acid, not glutathione, increased the intracellular concentration of BH₄. These data suggest that ascorbic acid enhances NO bioactivity in a BH₄-dependent manner by increasing intracellular BH₄ content.

Nitric oxide is produced from L-arginine in the vascular endothelium by the endothelial isoform of nitric-oxide synthase (NOS). Endothelial production of NO is crucial in the control of vascular tone (1), arterial pressure (2–4), smooth muscle cell proliferation (5, 6), and platelet adhesion to the endothelial surface (7). Impaired endothelium-derived NO bioactivity is a common feature of many vascular diseases (8–10) that is thought to contribute to their clinical manifestations (11, 12).

The action of NO is particularly sensitive to the local availability of superoxide. Both endothelial elaboration of NO and arterial relaxation in response to nitrovasodilators are dependent upon intact copper-zinc superoxide dismutase (SOD) activity (13, 14). Animal models of hypercholesterolemia (15, 16) and hypertension (17) demonstrate an excess vascular superoxide flux that is linked to reduced NO bioactivity. Conversely, increasing vascular SOD activity enhances NO-mediated arterial relaxation in experimental models of atherosclerosis (18, 19) and hypertension (17). Thus, scavenging superoxide has important implications for NO bioactivity under both normal and pathologic conditions.

Ascorbic acid also efficiently scavenges superoxide (20) and numerous studies in a host of pathologic conditions such as diabetes (21), hypercholesterolemia (22), smoking (23), and hypertension (24) indicate that NO bioactivity is improved by parenteral ascorbic acid at supraphysiologic concentrations (~10 mM). We have observed enhanced NO bioactivity in atherosclerotic patients after both acute (25) and chronic (1 month) oral ascorbic acid administration. With respect to these latter two observations, the role of superoxide scavenging is unclear. In particular, kinetic constraints indicate that extracellular ascorbic acid concentrations in these studies (~100 μM) cannot preserve NO bioactivity through superoxide scavenging (27). The purpose of this study, therefore, was to investigate the role of intracellular ascorbic acid on endothelial NO bioactivity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Medium M-199, minimal essential medium, fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Life Technologies, Inc. [3H]-arginine (10.2 Ci/mmol) and [14C]ascorbic acid (8 mCi/mmol) were obtained from NEN Life Science Products. Tetrahydrobipterin (BH₄) was from Research Biochemical International (Natick, MA). 2,3-Diaminophosphaleine was purchased from Molecular Probes (Eugene, OR), and Dowex AG 50WX-8 resin was from Bio-Rad. All other chemicals were obtained from Sigma. Solutions of authentic NO (~1 μM) were prepared in helium-deoxygenated distilled water as described (28).

**Cell Culture**—Porcine aortic endothelial cells (PAECs) were harvested from pig aorta using standard techniques and grown in M-199 supplemented with 15% FBS, penicillin, and streptomycin. Cells were grown in T75 flasks coated with fibronectin and passaged using calcium and magnesium-free Hanks’ balanced salt solution and trypsin-EDTA. Cultures were used up to passage 6 and exhibited typical endothelial cell morphology and positive staining for factor VIII-related antigens.

**Endothelial Ascorbic Acid**—For determination of ascorbic acid content, media or cell lysates were precipitated with an equal volume of 0.1 M diethylenetriamine phosphate (EDTA) and centrifuged. Supernatants were used to passaged 6 and exhibited typical endothelial cell morphology and positive staining for factor VIII-related antigens.
The slurry was washed twice with 20 volumes of 0.5M NaCl and 2 mM buffer A-equilibrated 2,5-ADP-Sepharose beads for at least 30 min. The lysate was mixed with 1 ml of lysis buffer with 1 mM CHAPS for 2 h. The protein concentration in the homogenate was centrifuged at 65,000 rpm for 60 min using a high-speed Eppendorf microcentrifuge (Boehringer-Mannheim). The supernatant was transferred onto nitrocellulose (Amersham Pharmacia Biotech). Membranes were incubated in phenol red-free minimal essential medium in the absence or presence of ascorbic acid. Nitric oxide synthesis was stimulated by 10 nM A23187 in HEPES-buffered PSS containing 200 μM CaCl2. The eluate typically contained <1% [3H]-arginine based upon parallel run controls without enzyme.

Intracellular GSH and Tetrahydrobiopterin—Intracellular GSH was estimated from the acid-soluble supernatant of PAECs lysed with 5% metaphosphoric acid/0.1 mM DTPA using an Ellman assay modified as described (35). Tetrahydrobiopterin was determined as described (36) with some modifications. PAECs from four 150-mm dishes were collected with trypsin-EDTA and lysed with pH 3 HPLC grade water containing 100 mM dithioerythritol and 100 μM DTPA, centrifuged at 13,000 × g for 10 min, and the supernatant and pellet were frozen immediately on dry ice and stored at −80 °C until analysis. Samples processed in this manner are stable for at least 1 year (36). For analysis, thawed samples were resolved with HPLC using a 25-cm LC18 reverse-phase column (Supelco, Bellefonte, PA) with a mobile phase of 50 mM HEPES-buffered physiologic salt solution (PSS) containing 22 mM HEPES, pH 7.4, 124 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.5 mM CaCl2, 0.16 mM HPO4, 0.4 mM H2PO4, 5 mM NaHCO3, 5.6 mM glucose, 10 mM NADPH in buffer B. Isolated eNOS was used immediately for experiments and typically demonstrated a specific activity for citrulline synthesis of ∼1.9 pmol/mg protein/30 min.

RESULTS

Endothelial Cell Ascorbic Acid Status and NO Bioactivity—Consistent with previous reports (37), we found that cultured cells from passages 2–6 contained undetectable levels of ascorbic acid by HPLC (<0.1 nmol/mg protein), likely because of low levels of ascorbate even in freshly obtained M199 (<0.1 μM). PAECs demonstrated dose- and time-dependent uptake of ascorbic acid from the media (Fig. 1, A and B) with a plateau in intracellular ascorbic acid after 5 h (14.7 ± 1.2 nmol/mg protein; n = 3) that was stable up to 24 h (Fig. 1A). Intracellular ascorbic acid also saturated at ∼20 nmol/mg protein with 75 μM ascorbic acid during a 5-h incubation (Fig. 1B). Calcium ionophore significantly increased endothelial cell cGMP from a basal level of 5.3 ± 0.8 pmol/mg protein to 58.2 ± 20.2 pmol/mg protein (n = 4, p < 0.01; data not shown). This increase in cGMP was inhibited ∼98% to 6.4 ± 1.9 pmol/mg protein (n = 3) in cells treated with 300 μM l-NAME (p < 0.01 for Student’s t test, data not shown). As shown in Fig. 1C, PAECs treated with ascorbic acid for 5 h demonstrated a dose-dependent increase in A23187-stimulated cGMP accumulation that closely paralleled ascorbic acid uptake (Fig. 1B). The maximum stimulation of A23187-induced cGMP accumulation with ascorbic acid was 170 ± 14% of the untreated control (Fig. 1C; p < 0.01 for ascorbic acid dose-response by ANOVA). To determine whether increased intracellular SOD activity would mimic the effect of ascorbic acid, we treated cells with Mn(III) tetrakis(4-benzoic acid)porphyrin (50 μM), a cell-permeable SOD mimic (38, 39), but did not observe any increase in A23187-stimulated cGMP accumulation (102.7 ± 8.4% of control).

To determine whether ascorbic acid altered the response to exogenous NO, we examined its effect on endothelial cGMP accumulation in response to authentic NO and sodium nitroprusside. PAECs incubated with 75 μM ascorbic acid for 5 h did not demonstrate any change in cGMP accumulation in response to either sodium nitroprusside (Fig. 2A) or authentic NO (Fig. 2B). Moreover, ascorbic acid also did not significantly...
alter the NO-independent accumulation of cGMP induced by atrial natriuretic peptide, an agonist of particulate guanylyl cyclase (Fig. 2C). Thus, the effect of ascorbic acid appears specific to eNOS-mediated endothelial cell cGMP accumulation.

Ascorbic Acid and Endothelial NO Production—To determine whether ascorbic acid enhances NO bioactivity as a function of NO production, we examined its effect on NO_2^- + NO_3^- (NOx) production from PAEC cultures. Stimulation of PAECs with 0.1 μM A23187 increased NOx approximately 72% from basal levels of 251 ± 40 pmol/10^6 cells/h to 433 ± 72 pmol/10^6 cells/h (Fig. 3A; p < 0.05). In PAECs treated with 75 μM ascorbic acid for 5 h, A23187-stimulated NOx was 290 ± 40 pmol/10^6 cells/h, an increase of 59% compared with 182 ± 45 pmol/10^6 cells/h in control cells (Fig. 3B; p < 0.05). Western blots of total cellular proteins revealed no significant effect of ascorbic acid on eNOS protein (Fig. 3C).

Ascorbic Acid and eNOS Enzymatic Activity—As shown in Fig. 4A, PAECs loaded with ascorbic acid exhibited a ~73% increase in eNOS enzymatic activity manifested as [3H]l-citrulline production from 251 ± 40 pmol/10^6 cells to 433 ± 72 pmol/10^6 cells (p < 0.001). There was no effect of ascorbic acid on the time-dependent uptake of [3H]l-arginine (data not shown). PAEC membrane fractions converted [3H]l-arginine to [3H]l-citrulline at a rate of 41.7 ± 2.1 pmol/mg/min. This activity was heat-stable and inhibited by >99% to 0.1 ± 0.4 pmol/mg/min by 200 μM l-NAME (Fig. 4B). Ascorbic acid over the intracellular concentration range (0.1–5 mM) significantly increased the rate of l-arginine conversion in PAEC membrane fractions (Fig. 4B), whereas copper-zinc SOD (0.01–1 mM) had no significant effect on eNOS activity in PAEC membranes (98 ± 2% of control, data not shown). Similar effects were seen using purified recombinant bovine eNOS (Fig. 4C).

Because we observed optimal stimulation of eNOS activity with 5 mM ascorbic acid, we examined its effects on enzyme kinetics using this concentration. As shown in Fig. 5A, the substrate dependence of eNOS demonstrated saturation with an upward shift in response to 5 mM ascorbic acid. Lineweaver-Burk analysis confirmed that ascorbic acid significantly increased eNOS V_{max} approximately 50% from 38.6 ± 0.6 nmol/ mg/min to 57.2 ± 4.7 nmol/mg/min (p < 0.05 by t test, n = 3) without altering the K_m (3.2 ± 0.3 versus 3.4 ± 0.5 μM, n = 3) for l-arginine (Fig. 5B).

Ascorbic Acid and BH_4—The activity of all NOS isoforms is dependent upon the presence of BH_4 that is bound to the enzyme in its physiologic state (40-43). We examined the role of BH_4 in the effect of ascorbic acid on eNOS activity. At concentrations of BH_4 below 10 μM, we observed a profound stimulatory effect of ascorbic acid (5 mM) on eNOS activity, whereas higher concentrations of BH_4 produced eNOS activity...
that was independent of ascorbic acid (Fig. 6A). Over four experiments, the concentration of half-maximal stimulation (EC50) for BH4 decreased from 0.31 ± 0.02 to 0.04 ± 0.01 μM (p < 0.05) in the presence of 5 mM ascorbic acid. Sepiapterin, a substrate for BH4 synthesis via the dihydrofolate reductase-dependent pterin salvage pathway (44), produced a dose-dependent reduction in the effect of ascorbic acid on A23187-induced cGMP accumulation in PAECs (Fig. 6B).

To determine the specificity of the ascorbic acid effect on NO bioactivity, we contrasted its effects with intracellular glutathione. Incubation of PAECs with buthionine sulfoximine (BSO) reduced GSH levels by 74% and had no effect on A23187-stimulated cGMP accumulation (Fig. 7A). Increasing GSH levels; 2.3-fold with glutathione ethyl ester likewise produced no
increase in eNOS activity manifested as intracellular cGMP in response to A23187 (Fig. 7A). In contrast, GSH did stimulate eNOS catalytic activity ~66% in vitro from 66.2 ± 8.5 mmol L-citrulline/mg/min to 110 ± 12.9 mmol L-citrulline/mg/min (Fig. 7B). Therefore, although both ascorbic acid and GSH enhance eNOS catalytic activity, only ascorbic acid enhances EDNO bioactivity in cultured PAECs.

Ascorbic Acid, GSH, and Intracellular BH₄—We next determined whether modulation of intracellular BH₄ explained the contrasting effects of ascorbic acid and GSH on NO bioactivity. As shown in Fig. 8, ascorbate-treated cells demonstrated a 226% increase in intracellular BH₄ compared with vehicle-treated cells (p < 0.05). In contrast, modulation GSH status with either BSO or GSH ethyl ester had no significant effect on intracellular BH₄. Thus intracellular ascorbic acid, but not GSH, is an important determinant of intracellular BH₄ concentration.

**DISCUSSION**

The major finding of this study is that intracellular ascorbic acid status is an important determinant of endothelial cell NO production, principally because of increased intracellular BH₄. We found that endothelial cells demonstrated enhanced eNOS-mediated cGMP accumulation as a function of increasing intracellular ascorbic acid (Fig. 1). We interpret this enhanced NO bioactivity with ascorbic acid to reflect an absolute increase in the amount of NO produced by endothelial cells. In support of this interpretation, ascorbic acid had no effect on the cGMP response to exogenous NO (Fig. 2), and endothelial cells containing ascorbic acid produced more L-citrulline and NO₂⁻ + NO₃⁻ than cells not containing ascorbate (Fig. 3). In addition, the enzymatic activity of eNOS was enhanced (via increased Vₘₐₓ) by ascorbic acid at concentrations that are physiologically relevant (0.1–5 mM). We also found that ascorbic acid enhanced eNOS catalysis in a manner that is dependent upon BH₄. In particular, the stimulatory effect of ascorbic acid on eNOS was lost with BH₄ concentrations exceeding 10 μM and in endothelial cells treated with sepiapterin, an agent that increases intracellular BH₄ levels (Fig. 6) (45–47). Although the effect of ascorbic acid on isolated eNOS was mimicked by GSH (Fig. 7B), we found that the effects of ascorbic acid and GSH on
endothelial cell NO bioactivity were distinct. In contrast to ascorbic acid, manipulation of intracellular GSH status had no effect on NO bioactivity. The mechanism for this distinction relates to our observation that intracellular ascorbic acid, but not GSH, appears to be an important determinant of endothelial cell BH4 content.

A potential role for reductants in NO production has been proposed for the neuronal isoform of NOS (nNOS). Komori and colleagues (48) found that nNOS purified in the absence of thiols was stimulated 4–7-fold by glutathione, dihydropteridine reductase, and ascorbic acid were interchangeable in stimulating nNOS activity principally as a result of an increase in the enzyme $V_{\text{max}}$ without any effect on L-arginine binding as the $K_m$ for L-arginine remained unchanged (Fig. 5). Thus, there is precedent for stimulation of NOS activity with ascorbic acid, and the mechanism for this effect is unrelated to superoxide or L-arginine.

We did observe that the effect of ascorbic acid was dependent upon the concentration of BH4 in both isolated enzyme preparations and intact cells (Fig. 6). In this regard, several aspects
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