THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 23, Issue of June 9, pp. 17399–17406, 2000
Printed in U.S.A.

Ascorbic Acid Enhances Endothelial Nitric-oxide Synthase Activity by Increasing Intracellular Tetrahydrobiopterin*

Received for publication, March 17, 2000
Published, JBC Papers in Press, April 3, 2000, DOI 10.1074/jbc.M002248200

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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 (NO_2^- + NO_3^-) 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 V_{max} by ~50% without altering the K_m for L-arginine. The effect of ascorbate was tetrahydrobiopterin (BH_4)-dependent, because ascorbate was ineffective with BH_4 concentrations >10 μM or in PAECs treated with sepiapterin to increase intracellular BH_4. 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 BH_4. These data suggest that ascorbic acid enhances NO bioactivity in a BH_4-dependent manner by increasing intracellular BH_4 content.

Nitric oxide is produced from L-arginine in the vascular endothelium by the endothelial isoform of nitric-oxide synthase (NOS).[1] 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) (26) 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. [^{3}H]L-arginine (10.2 Ci/mmol) and [^{14}C]ascorbic acid (8 mCi/mmol) were obtained from NEN Life Science Products. Tetrahydrobiopterin (BH_4) was from Research Biochemical International (Natick, MA). 2,3-Diaminonaphthalene was purchased from Molecular Probes (Eugene, OR), and Dowex AG 50W-X8 resin was from Bio-Rad. All other chemicals were obtained from Sigma. Solutions of authentic NO (~1 mM) 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, 10 μg/ml heparin sulfate, and antibiotics. 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 5% (v/v) metaphosphoric acid containing 0.1 mM diethylaminetriamine

* This work was supported by National Institute of Health Grants HL53398 and HL52936 (to J. A. V.) and HL59346 (to J. F. K). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: NOS, nitric-oxide synthase (EC 1.14.13.39); BH_4, tetrahydrobiopterin ((6*R*,6*S*)-2-amino-4-hydroxy-6-(L-erythro-1,2-dihydroxypropyl)-5,6,7,8-tetrahydropteridine); BSO, buthionine sulfoximine; cGMP, cyclic 3′,5′-guanosine monophosphate; CHAPS, 3-[(3-cholaminopropyl)dimethylammonio]-1-propane-sulfonate;DTPA, diethylaminetriamine pentaacetic acid; eNOS, endothelial nitric-oxide synthase; GSH, glutathione; L-NAME, N^G-nitro-L-arginine methyl ester; PAEC, porcine aortic endothelial cell; PSS, physiologic salt solution; SOD, superoxide dismutase; FBS, fetal bovine serum; HPLC, high pressure liquid chromatography; PBS, phosphate-buffered saline; ANOVA, analysis of variance; nNOS, neuronal NOS.

pentaactic acid (DTPA). Sample ascorbic acid content was determined using paired ion, reverse-phase HPLC with electrochemical detection as described (29). Uptake of ascorbic acid was determined using [^{14}C]L-ascorbic acid. PAECs were incubated with varying concentrations of [^{14}C]L-ascorbic acid, washed three times with PBS, and lysed in 60% methanol and 1 mM EDTA. Intracellular [^{14}C]L-ascorbic acid was determined by scintillation counting, and 100% of the intracellular radioactivity co-eluted with authentic L-ascorbic acid on paired ion, reverse-phase HPLC.

Assay of Endothelium-derived NO—We assayed endothelium-derived NO as the accumulation of cGMP in response to 1 μM A23187. Confluent PAECs in 12-well plates were equilibrated for 30 min in HEPES-buffered physiologic salt solution (PSS) containing 22 mM HEPES, pH 7.4, 124 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 0.16 mM HPO_4, 0.4 mM H_2PO_4, 5 mM $NaHCO_3$, 5.6 mM glucose, 10 μM indomethacin, and 200 μM 3-isobutyl-1-methylxanthine. Equilibrated cells were then stimulated with A23187 or vehicle for 5 min and lysed by the addition of 6% ice-cold trichloroacetic acid. Cell lysates were subjected to centrifugation at 13,000 $\times$ *g* for 10 min, and both the supernatant and pellet were stored at −70 °C until analysis. Determination of cGMP in supernatants was performed as described (30). The cell pellet protein content was determined by the BCA protein assay (Pierce) after solubilization with NaOH.

Endothelial Production of NO_2^- and NO_3^-—PAECs in 6-well plates 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 L-arginine for 8 h, and NO_2^- + NO_3^- was determined using the method of Miles and colleagues (31).

Western Blotting—PAECs in 60-mm dishes were incubated with or without ascorbic acid in M199/FBS for 5 or 24 h and lysed in 50 mM Tris-HCl, pH 7.5, containing 1% Nonidet P-40 and protease inhibitors. An aliquot of lysate was mixed with Laemmli buffer followed by boiling for 5 min. Cellular proteins (20 μg) were resolved on 7% SDS-PAGE and transferred onto nitrocellulose (Amersham Pharmacia Biotech). Membranes were incubated with a 1:2500 dilution of mouse mAb raised against human eNOS (Transduction Laboratories, Lexington, KY) followed by goat anti-mouse IgG peroxidase-conjugated secondary antibody and eNOS visualized using the ECL chemiluminescence kit (Amersham Pharmacia Biotech).

Expression and Purification of eNOS—The generation of baculovirus vector for expression of bovine eNOS has been described previously (32, 33). Briefly, Sf9 insect cells were infected at a multiplicity of infection of 5 in hemin chloride (1 μg/ml) containing Grace's medium. Cells were harvested 3 days after infection and lysed on ice for 30 min in buffer A (1% Triton X-100 in 50 mM Tris-HCl, pH 7.4, containing 1 mM phenylmethylsulfonyl fluoride and 1 μg/ml each aprotinin, leupeptin, soybean trypsin inhibitor, and pepstatin A). The lysate was mixed with 1 ml of buffer A-equilibrated 2′,5′-ADP-Sepharose beads for at least 30 min. The slurry was washed twice with 20 volumes of 0.5 M NaCl and 2 mM EGTA in buffer B (buffer A with 20% glycerol and no Triton X-100), and twice with 20 volumes of buffer B alone. Bound eNOS was eluted with 10 mM NADPH in buffer B. Isolated eNOS was used immediately for experiments and typically demonstrated a specific activity for citrulline production of 50–100 nmol/mg/min.

PAEC Membrane Preparations—Confluent PAECs in a T75 flask were suspended in PBS with a rubber policeman and washed twice with PBS. Harvested cells were sonicated in lysis buffer consisting of 50 mM Tris-HCl, pH 7.5, 100 μM DTPA, 10% glycerol and protease inhibitors. The homogenate was centrifuged at 65,000 rpm for 60 min using a Beckman Ti70 rotor. The pellet was then resolubilized by gentle rocking in lysis buffer with 1 mM CHAPS for 2 h. The protein concentration in solublized membrane preparations was determined by the BCA protein assay (Bio-Rad), and membrane fractions were prepared in this manner had no detectable (<1 μM) ascorbic acid by HPLC or nonprotein thiol by Ellman assay as described below.

[^{3}H]L-Arginine to [^{3}H]L-Citrulline Conversion—For intact cell assays, PAECs in 6-well plates were washed and incubated in PSS for 30 min followed by treatment with 200 μM L-arginine containing 25 μCi of [^{3}H]L-arginine. After 30 min, cells were stimulated with 1 μM A23187 for 15 min and lysed, and the lysate was subjected to anion exchange chromatography as described below. Total [^{3}H]L-arginine uptake was determined by liquid scintillation counting of total lysate aliquots prior to anion exchange chromatography and did not vary as a function of ascorbic acid status. Isolated enzyme assays (100 μl) contained 50 mM Tris, pH 7.5, 1 μM BH_4, 1 mM $CaCl_2$, 10 μg/ml calmodulin, 1 μM FAD, 1 μM FMN, 50 μM [^{3}H]L-arginine (~10^5 cpm), 0.5 mM NADPH, and 100 μM DTPA with or without added ascorbic acid. These conditions were chosen to minimize artificial eNOS superoxide production observed with higher concentrations (>1 μM) of flavins (34). Reactions were initiated by the addition of 0.1–0.25 μg of bovine eNOS or 150–200 μg of membrane protein and were stopped after 0–40 min at 37 °C by 1 ml of ice-cold stop buffer containing 20 mM sodium acetate, pH 5.5, 1 mM L-citrulline, 2 mM EDTA, and 2 mM EGTA. Samples were then applied to 0.8-ml Dowex AG50W-X8 columns pre-equilibrated with stop buffer. L-Citrulline was eluted twice with 1 ml of H_2O, and the 3-ml eluent was collected for determination of [^{3}H]L-citrulline by scintillation counting. Reactions were inhibited >95% by 200 μM L-NAME or the omission of $CaCl_2$. The eluate typically contained <1% [^{3}H]L-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 $\times$ *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 sodium acetate, 5.2 mM citrate, 60 μM EDTA, 160 μM dithioerythritol, and 5% methanol, pH 5.22. Quantification of BH_4 was accomplished with electrochemical detection at an applied potential of +0.12V using a Hewlett Packard series 1050 Chemstation. Under these conditions, biopterin and dihydrobiopterin are not detected.

Data Analysis—Values are presented as the means ± S.E. Dose response relationships were evaluated using one-way ANOVA and an appropriate *post hoc* comparison. Instances involving only two comparisons were evaluated with a Student's *t* test. Statistical significance was accepted if the null hypothesis was rejected with a $p < 0.05$.

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. 1*A*). Intracellular ascorbic acid also saturated at ~20 nmol/mg protein with 75 μM extracellular ascorbic acid during a 5-h incubation (Fig. 1*B*). 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 ($p < 0.01$ by Student's *t* test, data not shown). As shown in Fig. 1*C*, 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. 1*B*). The maximum stimulation of A23187-induced cGMP accumulation with ascorbic acid was 170 ± 14% of the untreated control (Fig. 1*C*; $p < 0.01$ 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. 2*A*) or authentic NO (Fig. 2*B*). Moreover, ascorbic acid also did not significantly

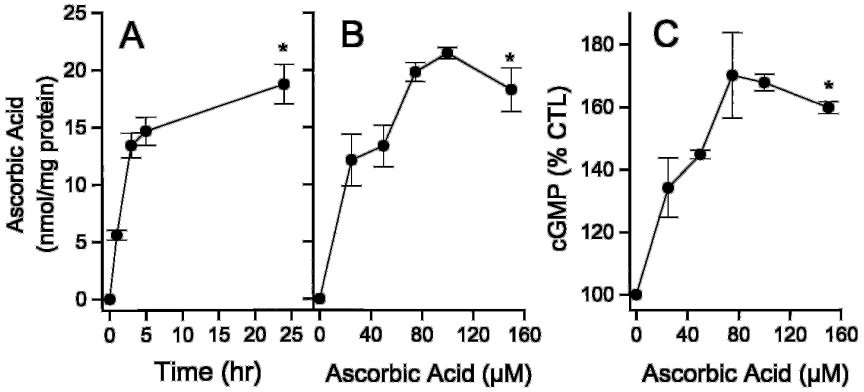

FIG. 1. **Endothelial cell uptake of ascorbic acid and its effect on NO-mediated cGMP accumulation.** Confluent PAECs in M199 with 1% FBS were incubated with 50 μM [^{14}C]L-ascorbic acid for the indicated time (*A*) or the indicated concentration of [^{14}C]L-ascorbic acid for 5 h (*B*). After incubation, cells were washed twice with PBS and lysed, and intracellular ascorbic acid was determined by scintillation counting. Data are the means ± S.E. derived from three experiments. *, $p < 0.01$ for effect of ascorbic acid by one-way ANOVA. *C*, PAECs were incubated as in *B*, washed with PBS, and incubated in HEPES-buffered PSS containing 200 μM 3-isobutyl-1-methylxanthine for 30 min. Cells were then stimulated with 1 μM A23187 (●) for 5 min, lysed with 6% trichloroacetic acid, and cGMP was determined as described under "Experimental Procedures". Data represent the percentage of change from cells treated without ascorbic acid and are the means ± S.E. of four experiments. *, $p < 0.01$ for dose-response of ascorbic acid by one-way ANOVA. PAEC cGMP in vehicle- and A23187-treated cells without ascorbic acid was 5.3 ± 0.8 and 58.2 ± 20.2 pmol/mg protein, respectively.

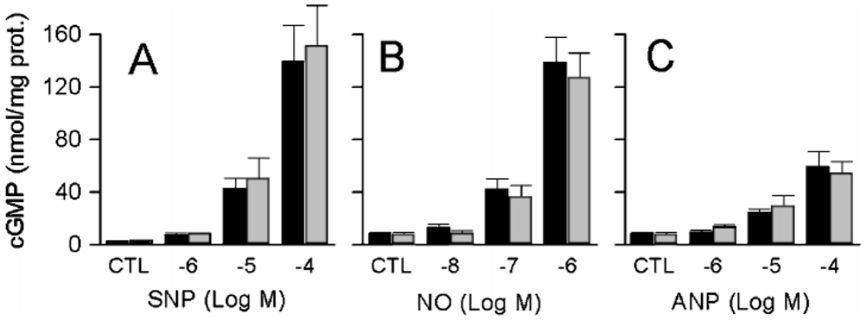

FIG. 2. **Ascorbic acid and eNOS-independent cGMP accumulation in endothelial cells.** Confluent PAECs were incubated with (*gray bars*) or without (*black bars*) 75 μM ascorbic acid for 5 h, washed with HEPES-buffered PSS and incubated with 200 μM 3-isobutyl-1-methylxanthine in HEPES-buffered PSS for 30 min. Cells were then treated with the indicated concentrations of sodium nitroprusside (*SNP*; *A*), NO (*B*), or atrial natriuretic peptide (*ANP*; *C*) for 2 min and lysed with 6% trichloroacetic acid, and cellular cGMP was determined as described under "Expermental Procedures". Data represent the means ± S.E. of three experiments.

alter the NO-independent accumulation of cGMP induced by atrial natriuretic peptide, an agonist of particulate guanylyl cyclase (Fig. 2*C*). 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 NO_x approximately 72% from basal levels of 251 ± 40 pmol/10^6cells/8 h to 433 ± 72 pmol/10^6cells/8 h (Fig. 3*A*; $p < 0.05$). In PAECs treated with 75 μM ascorbic acid for 5 h, A23187-stimulated NO_x was 290 ± 40 pmol/10^6cells/8 h, an increase of 59% compared with 182 ± 45 pmol/10^6cells/8 h in control cells (Fig. 3*B*; $p < 0.05$). Western blots of total cellular proteins revealed no significant effect of ascorbic acid on eNOS protein (Fig. 3*C*).

Ascorbic Acid and eNOS Enzymatic Activity—As shown in Fig. 4*A*, PAECs loaded with ascorbic acid exhibited a ~73% increase in eNOS enzymatic activity manifested as [^{3}H]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 [^{3}H]L-arginine (data not shown). PAEC membrane fractions converted [^{3}H]L-arginine to [^{3}H]L-citrulline at a rate of 41.7 ± 2.1 pmol/mg/min. This activity was heat-labile and inhibited by >99% to 0.1 ± 0.4 pmol/mg/min by 200 μM L-NAME (Fig. 4*B*). Ascorbic acid over the intracellular concentration range (0.1–5 mM) significantly increased the rate of L-arginine conversion in PAEC membrane fractions (Fig. 4*B*), whereas copper-zinc SOD (0.01–1 μM) 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. 4*C*).

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. 5*A*, 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. 5*B*).

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

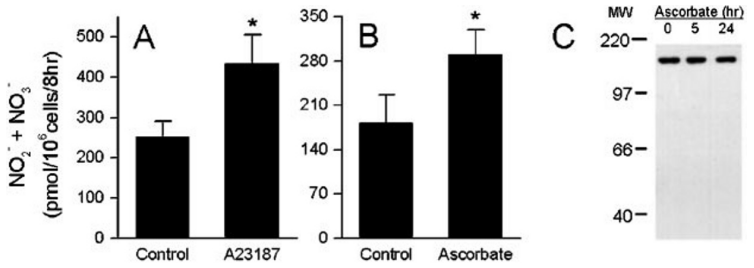

FIG. 3. **Effect of ascorbic acid on NO production and eNOS protein in endothelial cells.** *A*, confluent PAECs were incubated with 10 nM A23187 or vehicle in HEPES-buffered PSS for 8 h and $NO_2^- + NO_3^-$ determined as described under "Experimental Procedures." Data represent the means ± S.E. from five experiments; *, $p < 0.05$ *versus* control by two-tailed *t* test. *B*, confluent PAECs in phenol-red free minimal essential medium with 1% FBS were incubated with or without 75 μM ascorbic acid for 18 h, washed with HEPES-buffered PSS, and incubated with A23187 (10 nM) for 8 h. After incubation, $NO_2^- + NO_3^-$ was determined as in *A*. Data represent the means ± S.E. from five experiments; *, $p < 0.05$ *versus* control two-tailed *t* test. *C*, confluent PAECs in phenol red-free minimal essential medium containing 1% FBS were incubated with 75 μM ascorbic acid for the indicated times. Cells were then lysed, proteins were resolved with 7% SDS-PAGE, and Western blots were performed as described under "Experimental Procedures" with anti-eNOS antibody (Transduction Labs). One blot representative of three is shown. *MW*, molecular mass (kDa).

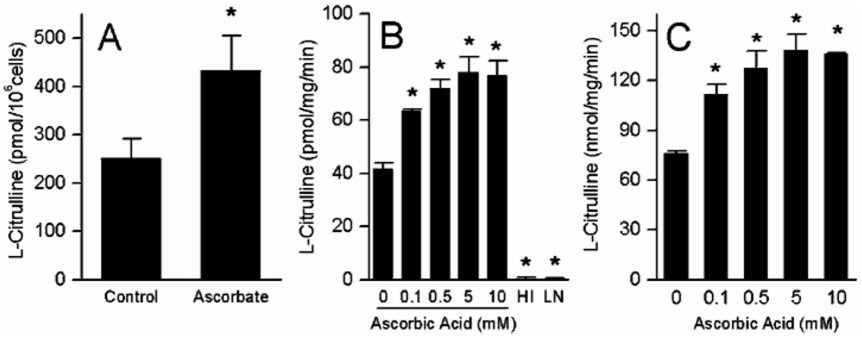

FIG. 4. **Effect of ascorbic acid on eNOS enzymatic activity.** *A*, intact PAECs were incubated with 75 μM ascorbic acid or vehicle for 5 h and washed, and [^{3}H]L-citrulline production was determined as described under "Experimental Procedures." $p < 0.001$ *versus* control by two-tailed *t* test. PAEC membrane fractions (*B*; 150–200 μg of protein) or recombinant bovine eNOS (*C*; 0.1–0.25 μg of protein) were incubated as described under "Experimental Procedures." After 30 min at 37 °C, reactions were terminated by 1 ml of ice-cold stop buffer and applied to 0.8 ml of Dowex AG50W-X8 columns, and [^{3}H]L-citrulline in the eluent was determined by scintillation counting. Some incubations contained heat-inactivated (*HI*) membrane fractions (5 min boiling) or 200 μM L-NAME (*LN*). Data represent the means ± S.E. of five independent experiments. *, $p < 0.05$ *versus* 0 mM ascorbic acid by one way ANOVA with a Newman-Keuls comparison.

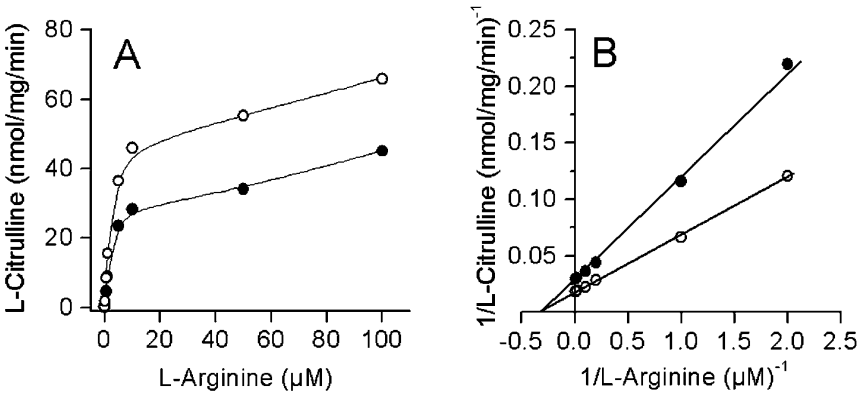

FIG. 5. **Effect of ascorbic acid on eNOS kinetics.** *A*, purified bovine eNOS (0.1–0.25 μg of protein) was incubated as in Fig. 4 with the indicated concentration of [^{3}H]L-arginine (~10^5 cpm) in the presence (○) or absence (●) of 5 mM ascorbic acid. After 15 min at 37 °C, reactions were terminated, and [^{3}H]L-citrulline in the eluent was determined by scintillation counting as described under "Experimental Procedures." *B*, Lineweaver-Burk plot of the data in *A*. Data are derived from one experiment performed in duplicate representative of five.

that was independent of ascorbic acid (Fig. 6*A*). Over four experiments, the concentration of half-maximal stimulation (EC_{50}) for BH_4 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 BH_4 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. 6*B*).

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. 7*A*). Increasing GSH levels ~2.3-fold with glutathione ethyl ester likewise produced no

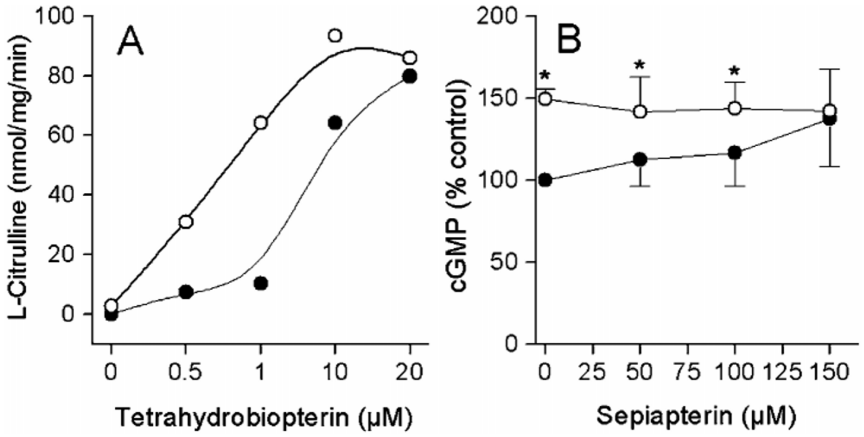

FIG. 6. **Effect of ascorbic acid on eNOS activity as a function of BH_4.** *A*, purified bovine eNOS (0.1–0.25 μg of protein) was incubated as described under "Experimental Procedures" with the indicated concentration of BH_4 in the presence (○) or absence (●) of 5 mM ascorbic acid. After 15 min at 37 °C, reactions were terminated, and [^{3}H]L-citrulline in the eluent was determined by scintillation counting. Data are derived from three experiments. *B*, confluent PAECs in M199/6% FBS were incubated with the indicated concentrations of sepiapterin with (○) or without (●) 75 μM ascorbic acid for 5 h and washed with HEPES-buffered PSS, and cellular cGMP was determined in response to 1 μM A23187 as described under "Experimental Procedures." Data are expressed as a percentage of eNOS activity without ascorbic acid or sepiapterin and represent the means ± S.E. of six experiments. *, $p < 0.05$ *versus* without ascorbic acid by two-way repeated measures ANOVA with a Newman-Keuls comparison.

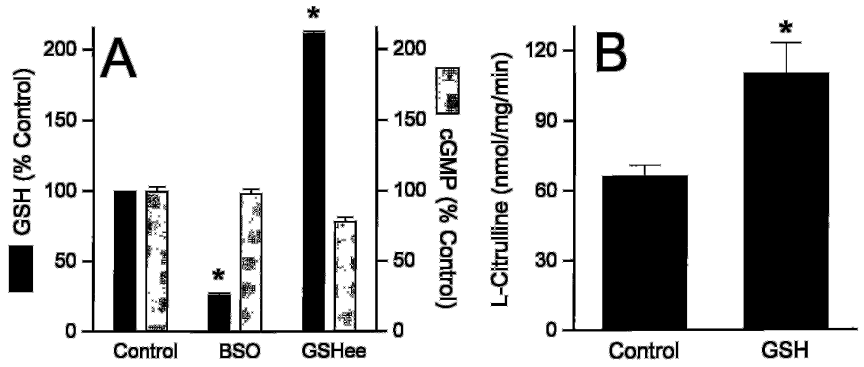

FIG. 7. **Effect of GSH manipulation on NO bioactivity.** *A*, confluent PAECs in M199 with 1% FBS were incubated with BSO (150 μM for 16 h), GSH ethyl ester (*GSHee*; 20 mM for 2 h) or vehicle alone (control). PAEC were then washed with HEPES-buffered PSS and GSH content, and cGMP response to 1 μM A23187 were determined as described under "Experimental Procedures." Control cell GSH content was 27.4 nmol/mg protein, and the control cGMP response to 1 μM A23187 was 58.2 ± 20.2 pmol/mg protein. *B*, bovine eNOS (0.1–0.25 μg of protein) was incubated as described in the legend to Fig. 4 for 15 min with or without 7 mM GSH, and [^{3}H]L-citrulline production was determined as described under "Experimental Procedures." Data are the means ± S.E. from five experiments. *, $p < 0.05$ *versus* control by one-way ANOVA with a *post hoc* Dunnet's test (*A*) or Student's *t* test (*B*).

increase in eNOS activity manifested as intracellular cGMP in response to A23187 (Fig. 7*A*). In contrast, GSH did stimulate eNOS catalytic activity ~66% *in vitro* from 66.2 ± 4.5 nmol L-citrulline/mg/min to 110 ± 12.9 nmol L-citrulline/mg/min (Fig. 7*B*). 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 BH_4—We next determined whether modulation of intracellular BH_4 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 BH_4 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 BH_4. Thus intracellular ascorbic acid, but not GSH, is an important determinant of intracellular BH_4 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 BH_4. 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 NO_2^- + NO_3^- than cells not containing ascorbate (Fig. 3). In addition, the enzymatic activity of eNOS was enhanced (via increased V_{max}) 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 BH_4. In particular, the stimulatory effect of ascorbic acid on eNOS was lost with BH_4 concentrations exceeding 10 μM and in endothelial cells treated with sepiapterin, an agent that increases intracellular BH_4 levels (Fig. 6) (45–47). Although the effect of ascorbic acid on isolated eNOS was mimicked by GSH (Fig. 7*B*), we found that the effects of ascorbic acid and GSH on

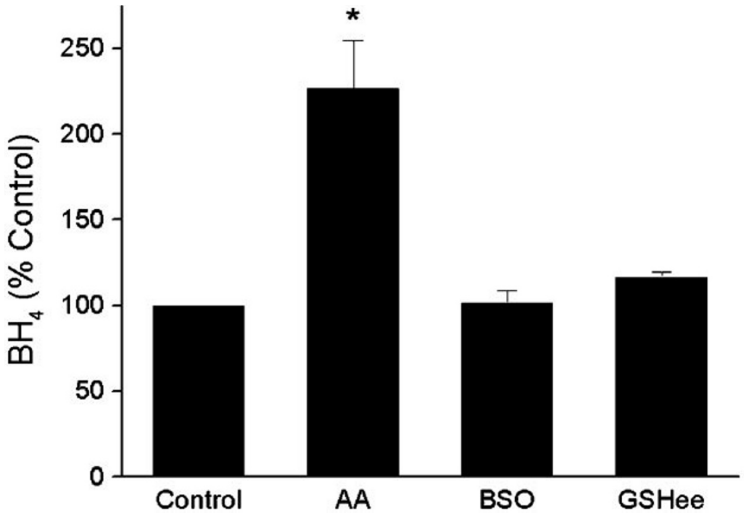

FIG. 8. **Effect of ascorbic acid and GSH manipulation on intracellular tetrahydrobiopterin.** Confluent PAECs in M199/1% FBS were incubated with ascorbic acid (75 μM for 5 h), BSO (150 μM for 16 h), GSH ethyl ester (*GSHee*; 2 mM for 2 h) or vehicle alone (control). Cells were washed, and intracellular tetrahydrobiopterin was determined by HPLC with electrochemical detection as described under "Experimental Procedures." Data are the means ± S.E. from six or seven experiments. Control PAECs contained 10.0 ± 4.1 pmol of BH_4/mg of protein. *, $p < 0.05$ *versus* control by one-way ANOVA on ranks.

TABLE I
Ascorbic acid-sensitive enzymes

Enzyme	Function	Mechanism	Reference(s)
Prolyl-4-hydroxylase (EC 1.14.11.2)	Trans-4-hydroxylation of proline in procollagen biosynthesis	Reduction of iron with increased V_{max}	57 and 58
Lysine hydroxylase (EC 1.14.11.4)	5-Hydroxylation of lysine in procollagen biosynthesis	Reduction of iron with increased V_{max}	59 and 60
4-Hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27)	Hydroxylation and decarboxylation of hydroxyphenylpyruvate in tyrosine metabolism	Reduction of iron with increased V_{max}	61
Dopamine β-monooxygenase (EC 1.14.17.1)	Dopamine β-hydroxylation in norepinephrine biosynthesis	Source of electrons with increased V_{max}	62
Peptidylglycine monooxygenase (EC 1.14.17.3)	Hydroxylation of glycine-extended peptides for peptide hormone processing	Source of electrons with increased V_{max}	63
Phenylalanine 4-monooxygenase (EC 1.14.16.1)	Hydroxylation of phenylalanine to form tyrosine	Increased V_{max}, possible reduction of dihydrobiopterin	66
Tyrosine 3-monooxygenase (EC 1.14.16.2)	Hydroxylation of tyrosine in catecholamine biosynthesis	Increased V_{max}, possible reduction of dihydrobiopterin	64

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 BH_4 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, cysteine, dithiothreitol, and β-mercaptoethanol. Because a similar effect was observed for dihydropteridine reductase, the authors proposed a mechanism involving the reduction of dihydrobiopterin to BH_4. In that study, ascorbic acid was not an effective replacement for thiols (48). This latter finding contrasts with that of Hofmann and Schmidt (49), who demonstrated that glutathione, dihydropteridine reductase, and ascorbic acid were interchangeable in stimulating nNOS activity. The latter two reagents, however, were not effective in preventing nNOS inactivation during catalysis. Thus, there is precedent for stimulation of NOS activity in the presence of reducing agents.

The data presented here extend these previous studies to eNOS, demonstrating that ascorbic acid at a concentration of 5 mM stimulated bovine eNOS enzymatic activity ~80%, a value that is in excellent agreement with its ~70–90% stimulatory effect on nNOS (49). Our results are also in excellent agreement with a recent report by Heller and colleagues (50) who found that human endothelial cells treated with ascorbic acid demonstrated enhanced NO production as determined by L-citrulline formation and cGMP accumulation in response to both ionomycin and thrombin. Using a number of ascorbic acid derivatives, these authors found that the reductive capacity of ascorbic acid was an important component of its action and that ascorbate had no effect on L-arginine uptake, an important determinant of endothelial NO bioactivity (51). Our data provide some additional mechanistic insight into the action of ascorbic acid. We were unable to duplicate the effect of ascorbic acid with physiologic concentrations of SOD, suggesting that superoxide scavenging by ascorbate is not involved in eNOS stimulation. This finding is consistent with our report demonstrating that ascorbic acid is a poor substitute for SOD (27). Moreover, we observed that ascorbic acid stimulated eNOS activity principally as a result of an increase in the enzyme V_{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 BH_4 in both isolated enzyme preparations and intact cells (Fig. 6). In this regard, several aspects

of BH_4 warrant consideration. Only the completely reduced (tetrahydro) form of biopterin supports NOS coupling of NADPH oxidation to NO synthesis (52). Because NOS catalysis is associated with oxidation of BH_4 (53, 54) and ascorbic acid has been proposed to maintain BH_4 in a reduced state (55), one might speculate that ascorbic acid simply maintains BH_4 in its reduced state. Indeed, our experiments with bovine eNOS from Sf9 cells would tend to support such an argument because ascorbic acid was ineffective with saturating BH_4 levels and GSH could substitute for BH_4. However, this line of reasoning would also predict equivalent effects on NO bioactivity with the intracellular manipulation of either ascorbic acid or GSH, a prediction clearly inconsistent with the data presented here (Figs. 1 and 7). The major distinction between intracellular GSH and ascorbic acid manipulation in this study was the activity of ascorbic acid alone to increase endothelial cell BH_4 content. The precise mechanism for this observation is not yet clear but may relate to the prevention of intracellular BH_4 oxidation or some effect of ascorbate on BH_4 synthesis. Further investigation will be required to clearly define the role of ascorbic acid in maintaining intracellular BH_4.

In the context of other ascorbate-sensitive enzymes, the fact that ascorbic acid stimulates NO synthesis is not particularly surprising. The first step in NO synthesis involves the hydroxylation of L-arginine to *N*-hydroxy-L-arginine (56), and several enzymes that mediate amino acid hydroxylation are ascorbate-sensitive (Table I). In the first three instances, ascorbic acid acts through a reductive mechanism maintaining the iron cofactor in its reduced state for the hydroxylation of proline (57, 58), lysine (59, 60), and 4-hydroxyphenylpyruvate (61). In the copper-containing enzymes dopamine β-hydroxylase and peptidylglycine monooxygenase, ascorbic acid serves as a source of electrons for reduction of molecular oxygen (62, 63). In contrast, the mechanism of ascorbate action with tyrosine 3-hydroxylase is less clear. Like the NOS isoforms, this enzyme utilizes tetrahydrobiopterin as a cofactor, and one suggested mechanism of ascorbate action involves maintaining the pterin in a reduced state (64). Whether ascorbic acid stimulates NO synthesis principally through facilitating L-arginine hydroxylation remains to be determined.

The effects of ascorbic acid described here are physiologically relevant based upon considerable experience with ascorbic acid and NO bioactivity in human subjects. Patients with atherosclerosis treated both acutely (25, 26) or chronically (26) with oral ascorbic acid demonstrate enhanced endothelium-derived NO bioactivity. In fact, ascorbic acid improves endothelial NO bioactivity in a number of disease states including diabetes mellitus (21), hypercholesterolemia (22), heart failure (65), and hypertension (24). Given the fundamental role of intracellular BH_4 for NO synthesis (46), the BH_4-dependent effect of ascorbic acid described here provides an attractive explanation for such uniform clinical observations.

In summary, the data presented here indicate that endothelial cell NO bioactivity is sensitive to intracellular ascorbic acid. In particular, endothelial cell production of NO is enhanced by intracellular ascorbate, and this effect appears to be mediated through an effect on intracellular BH_4. These data suggest a novel mechanism for numerous *in vivo* observations that endothelium-derived NO bioactivity is enhanced by ascorbic acid.

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