L-Ascorbic Acid Potentiates Nitric Oxide Synthesis in Endothelial Cells

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Ascorbic acid has been shown to enhance impaired endothelium-dependent vasodilation in patients with atherosclerosis by a mechanism that is thought to involve protection of nitric oxide (NO) from inactivation by free oxygen radicals. The present study in human endothelial cells from umbilical veins and coronary arteries investigates whether L-ascorbic acid additionally affects cellular NO synthesis. Endothelial cells were incubated for 24 h with 0.1–100 μM ascorbic acid and were subsequently stimulated for 15 min with ionomycin (2 μM) or thrombin (1 unit/ml) in the absence of extracellular ascorbate. Ascorbate pretreatment led to a 3-fold increase of the cellular production of NO measured as the formation of its co-product citrulline and as the accumulation of its effector molecule cGMP. The effect was saturated at 100 μM and followed a similar kinetics as seen for the uptake of ascorbate into the cells. The investigation of the precursor molecule L-gulonolactone and of different ascorbic acid derivatives suggests that the end niel structure of ascorbate is essential for its effect on NO synthesis. Ascorbic acid did not induce the expression of the NO synthase (NOS) protein nor enhance the uptake of the NOS substrate L-arginine into endothelial cells. The ascorbic acid effect was minimal when the citrulline formation was measured in cell lysates from ascorbate-pretreated cells in the presence of known cofactors for NOS activity. However, when the cofactor tetrahydrobiopterin was omitted from the assay, a similar potentiating effect of ascorbate pretreatment as seen in intact cells was demonstrated, suggesting that ascorbic acid may either enhance the availability of tetrahydrobiopterin or increase its affinity for the endothelial NOS. Our data suggest that intracellular ascorbic acid enhances NO synthesis in endothelial cells and that this may explain, in part, the beneficial vascular effects of ascorbic acid.

Endothelium-derived nitric oxide (NO) exerts several vasoprotective activities including smooth muscle relaxation, inhibition of platelet activation, and regulation of endothelial cell permeability and adhesivity (1–4). NO is generated from the conversion of L-arginine to L-citrulline by the enzymatic action of an NADPH-dependent NO synthase (NOS) that requires tetrahydrobiopterin, FAD, and FMN as cofactors (5). The endothelial NOS isoform (ecNOS) is constitutively expressed and is activated upon an increase of intracellular calcium following cell stimulation with receptor-dependent stimuli such as thrombin and bradykinin or with receptor-independent stimuli like calcium ionophore (6).

Since NO interferes with key processes in atherogenesis (7), a lack of NO might promote the development of atherosclerosis. Indeed, clinical studies have confirmed an impairment of vascular NO synthesis in patients with atherosclerosis or with increased atherogenic risk factors (8). The dysfunction of the endothelial NO pathway may involve impaired signal transduction mechanisms, decreased ecNOS activity, reduced intracellular availability of L-arginine, or increased inactivation of NO by superoxide anions or oxidized low density lipoproteins (7, 8). Accordingly, initial therapeutic strategies for an improvement of endothelial vasodilator function include the application of angiotensin-converting enzyme inhibitors that stimulate NO synthesis through the local accumulation of bradykinin as well as the supplementation of L-arginine and the use of antioxidants (7, 9).

Ascorbic acid is the most important water-soluble antioxidant in human plasma (10). It effectively scavenges superoxide and other reactive oxygen species and protects lipids against peroxidation (11, 12). Ascorbic acid is thought to play a protective role in atherogenesis since epidemiological studies have demonstrated that plasma ascorbic acid levels are inversely related to the mortality from coronary heart disease (13). Moreover, a number of conditions known to be associated with an increased risk for atherosclerosis have been linked with lower plasma levels of ascorbic acid (14–17). Recently, several studies have shown that an acute application of ascorbic acid enhanced endothelium-dependent vasodilation in patients with diabetes, coronary artery disease, hypertension, hypercholesterolemia, or chronic heart failure and in cigarette smokers (18–23). These findings were attributed to the radical scavenging ability of ascorbic acid which may protect NO from inactivation. So far, however, direct effects of ascorbic acid on endothelial NO synthesis have not been examined.

The present study was designed to investigate whether ascorbic acid affects NO production in human endothelial cells. The synthesis of NO was measured as the formation of its co-product citrulline and as the accumulation of its effector molecule cGMP after stimulating ascorbate-pretreated cells with the calcium-increasing agonists ionomycin and thrombin. Additionally, the influence of ascorbic acid on calcium-dependent citrulline formation in cell lysates and on the content of ecNOS protein was investigated. We report that ascorbic acid stimulates NO synthesis in endothelial cells, and we suggest...
that this action may be an important characteristic of this agent to exert its beneficial effects in the vascular system.

**EXPERIMENTAL PROCEDURES**

**Materials**—Plasticware for cell culture was from Greiner Labortechnik (Frickenhausen, Germany). Medium 199 (M199), human serum, fetal calf serum, collagenase, and human serum albumin (HSA) were from Boehringer Ingelheim Bioproducts (Heidelberg, Germany). L-[2,3,4,5-4H]Arginine monohydrochloride (61 Ci/mmole), L-[U-14C]arginine monohydrochloride (303 Ci/mmole), L-[14C]ascorbic acid (16 Ci/mmole), [H]cGMP Biotra radioimmunoassay systems, hyperfilm βmax, ECL, and hyperfilm ECL were purchased from Amersham Corp. (Bucks, UK). Tran35S-label and methionine-free RPMI medium were from ICN Pharmaceuticals (Costa Mesa, CA); nitrocellulose was from Millipore (Eschborn, Germany); specific monoclonal antibodies against human ecNOS (clone 3) or against murine macrophage-inducible NO (iNOS, clone 6) were from Transduction Laboratories (Lexington, KY); the fluorescent isothiocyanate-conjugated secondary antibody (rabbit anti-mouse IgG (H + L)) with minimal cross-reaction to human serum proteins was from Dianova (Hamburg, Germany); NADPH, tetrahydrobiopterin, and L-nitroarginine methyl ester (lNAME) were from Alexis Corp. (Laufelfingen, Switzerland); Endothelial cell NOS (eNOS) substrate, pervanadate-labeled anti-mouse (Fab specific), anti-mouse-agarose, FAD, FNM, calmodulin, iomyacin, thrombin, EDTA, EGT, trypsin/EDTA solution (0.05/0.02%, v/v), leupeptin, phenylmethylsulfonyl fluoride (PMSF), L-aspartic acid, dehydro-L-ascorbic acid, ascorbate 2-phosphate, ascorbate 2-sulfate, l-gulonolactone, and other reagents were purchased from Sigma (Deisenhofen, Germany).

The composition of the Hepes buffer (pH 7.4) was as follows (in mM): 10 Hepes, 145 NaCl, 5 KCl, 1 MgSO4, 10 glucose, 1.5 CaCl2. Hepes homogenization buffer (pH 7.2) consisted of (in mM) 20 Hepes, 0.5 EDTA, 0.5 EGTA, 1 dithiothreitol (DTT) 1 PMSF, 0.001 pepstatin, and 0.002 leupeptin. Hepes/sorbitol buffer (pH 7.4) contained (in mM) 10 Hepes, 340 sorbitol, 1 EDTA, 2 DTT, 1 PMSF, 0.6 leupeptin, and 0.025 pepstatin. The composition of the solubilization buffer was 100 mM NaCl, 1% NP-40 and 1% SDS.

**Cell Cultures**—Human umbilical cord vein endothelial cells (HUVEC) were prepared with 0.05% collagenase, and primary coronary artery endothelial cells (CAEC) were obtained from Clonetics (San Diego, CA). Cells were cultured in 75-cm² plastic flasks in M199 containing 15% fetal calf serum, 5% human serum, and 7.5 µg/ml endothelial cell growth supplement. Confuent cultures were detached by trypsin/EDTA and suspended on 30-mm diameter wells for the measurement of NO production and determination of the other parameters. Experiments were carried out with monolayers of the first to second passage (HUVEC) or third to fourth passage (CAEC).

**Experimental Incubations**—Preincubations of HUVEC or CAEC with L-arginine, acetyl-L-citrulline, dehydroascorbic acid, and different ascorbic acid derivatives were performed in culture medium for 1–24 h. The low content of ascorbic acid in M199 (0.3 µM) was neglected since the liquid media were stored between 1 and 3 weeks before use, and the compound is extremely labile in solution. L-Ascorbic acid stock solution containing 15% fetal calf serum, 5% human serum, and 7.5 µg/ml endo
telial cell growth supplement were prepared in 1.5 ml of Hepes buffer (pH 7.4) containing 100 µM L-arginine or L-citrulline or 1 unit/ml thrombin. After 15 min the reaction was stopped with cold phosphate-buffered saline (PBS) containing 5 mM Hepes-Na (pH 5.5) and applied to 2-ml columns of Dowex AG-50W-X8 (Na+ form). The [3H]citrulline content of the eluate was quantified by liquid scintillation counting. Agonist-induced citrulline production was calculated from the difference in radioactivity from ionomycin- or thrombin-stimulated cells and the corresponding unstimulated cells and was expressed in pmoles/kg cell protein. Basal citrulline synthesis was determined from the l-NAME (1 mM, 30 min preincubation)-inhibitable radioactivity in unstimulated cells and was not always detectable.

**Determination of cGMP**—HUVEC monolayers were incubated for 30 min in M199 containing 0.25% HSA and 0.5 mM isobutylmethylxanthine. Subsequently, the cells were stimulated with 2 µM ionomycin or 1 unit/ml thrombin for 15 min. The reaction was stopped with 96% ethanol. When the ethanol had evaporated, 0.3 ml of buffer (50 mM Tris, 4 mM EDTA (pH 7.5)) was applied. The cGMP content of 100 µl of the supernatant was measured by radioimmunoassay following the instructions of the manufacturer. The intracellular cGMP concentration was expressed in pmoles/ml cell protein. The agonist-induced cGMP production was determined from the difference of cGMP content in ionomycin- or thrombin-stimulated cells and the corresponding unstimulated cells.

**[14C]Ascorbic Acid Uptake in Endothelial Cells**—HUVEC were incubated in culture medium containing 100 µM [14C]ascorbic acid (16 Ci/mmol). After various time periods, incubations were stopped by washing the cells with cold Hepes buffer (pH 7.4) containing 100 µM phosphatase which had been shown to prevent the efflux of [14C]dehydroascorbic acid (25). HUVEC were lysed with solubilization buffer; an aliquot of the lysate was taken to determine the protein content (26), and the radioactivity of the remaining sample was measured by liquid scintillation counting.

**[14C]Arginine Uptake into Endothelial Cells**—HUVEC were incubated in culture medium containing 335 µM l-[14C]arginine (3 mCi/mmol). After various time periods, incubations were stopped by washing the cells with cold Hepes buffer (pH 7.4) containing 5 mM l-arginine. The solubilization of the cells and the measurement of radioactivity were performed as described above.

**Electrophoresis and Immunoblotting**—HUVEC were detached by trypsin/EDTA and suspended in a small volume of Hepes/sorbitol buffer (pH 7.4). For preparation of subcellular fractions, cell suspensions were sonicated on ice and centrifuged for 60 min at 100,000 × g and 4 °C. Proteins were solubilized by boiling the cells as well as the soluble and particulate fractions in Laemmli sample buffer. The samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 7% gels (50 µg of protein per lane), blotted onto nitrocellulose membranes, and subsequently subjected to immunostaining with primary antibodies against human ecNOS or against murine iNOS (1:250, 1.5 h). After an incubation with a peroxidase-conjugated secondary antibody (1:1000, 1.5 h), visualization of NOS was achieved using the ECL technique.

**Flow Cytometric Measurement of ecNOS Protein**—HUVEC were fixed with 0.5% formaldehyde in PBS for 2.5 min, disassociated by a combination of trypsinization and pipetting to suspensions of trypsinized cells that were finally resuspended in PBS containing 0.1% HSA. Samples of 5 × 10⁶ cells/50 µl were labeled with a monoclonal antibody to ecNOS (1:25, 30 min) in the presence of 0.06% saponin (w/v) and subsequently incubated with fluorescein isothiocyanate-conjugated anti-mouse IgG (H + L) (1:50, 30 min). The cell-associated fluorescence of 5000 cells per sample was determined in a FACScan flow cytometer (Becton/Dickinson) using the CellQuest software and reported as mean fluorescence intensity in the FL1 channel. The mean fluorescence intensity values were corrected for nonspecific staining. Calibration of the flow cytometer and calculation of the numbers of ecNOS molecules per cell from the corrected mean fluorescence intensity values were done using Dako FluoroSpheres.

**Metabolic Labeling and Immunoprecipitation of ecNOS**—HUVEC were incubated with 100 µM ascorbic acid for 6 h. Subsequently, 50 µCi of [3H]arginine was added to the culture medium, and the cells were subsequently subjected to immunostaining with primary antibodies against human ecNOS or against murine iNOS (1:250, 1.5 h). After an incubation with a peroxidase-conjugated secondary antibody (1:1000, 1.5 h), visualization of NOS was achieved using the ECL technique.
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RESULTS

Effect of Ascorbic Acid on Citrulline and cGMP Formation in Endothelial Cells—NO production upon endothelial cell stimulation is accompanied by an increased synthesis of citrulline which is produced stoichiometrically with NO, and by an accumulation of intracellular cGMP which is generated when NO activates the soluble guanylate cyclase of the cells. Accordingly, both parameters indicate the formation of NO. Stimulation of HUVEC with ionomycin led to a citrulline formation of 3.7 ± 0.3 pmol/mg cell protein (n = 6). 24 h preincubation of HUVEC with 0.1–100 μM ascorbic acid potentiated both ionomycin- and thrombin-stimulated cGMP formation up to 2.9- and 2.7-fold, respectively, thus confirming the effects on citrulline formation (Fig. 2). Again, the agonist-stimulated cGMP increase in both untreated and ascorbic-acid-preincubated cells was entirely blocked by a 30-min preincubation with 1 mM l-NAME. Ascorbic acid did not affect the basal cGMP formation that was 1.9 ± 0.2 pmol/mg in control cells and 2.0 ± 0.4 pmol/mg in cells pretreated with 100 μM ascorbic acid for 24 h (n = 20).

Time Dependence of the Ascorbic Acid Effect—Preincubation of HUVEC with 100 μM l-ascorbic acid for different periods followed by stimulation with ionomycin revealed that the potentiating effect on citrulline and cGMP formation was related to the incubation time. A maximal amplification of ionomycin-induced citrulline and cGMP production was achieved after an 18-h pretreatment (Fig. 3). After a 6-h preincubation, 46 and 30% of the maximal effects on citrulline and cGMP synthesis, respectively, were measured, whereas a 1-h treatment with ascorbic acid did not cause any potentiation of calcium-dependent citrulline and cGMP formation (Fig. 3).

Transport of Ascorbic Acid into Endothelial Cells—To measure the [14C]ascorbic acid uptake into HUVEC, cells were incubated with 100 μM [14C]ascorbate (16 mCi/mmol) for 1–24 h. About 17, 36, 47, and 75% of the maximal ascorbic acid uptake were achieved after 1, 2, 3, and 6 h, respectively, while saturation occurred between 12 and 24 h (Fig. 4). At 24 h the cellular ascorbic acid level calculated from the specific radioac-
Incubated with 100 μM ionomycin-induced citrulline and cGMP formation. Data are shown as counts/min of incorporated [14C]ascorbic acid radioactivity of cell lysates was measured by liquid scintillation counting for the indicated times, washed, and subsequently solubilized. The activity of the added compound was 21.5 ± 3.7 nmol/mg protein assuming that ascorbate in non-supplemented cells was negligible (27).

Effect of Ascorbic Acid Derivatives on Citrulline and cGMP Formation in Endothelial Cells—In addition to L-ascorbic acid, we investigated how the ascorbate precursor molecule L-gulonolactone, the oxidation product dehydro-L-ascorbic acid, as well as the derivatives ascorbate 2-phosphate and ascorbate 2-sulfate (Fig. 5) influence ionomycin-stimulated NO production. All compounds were applied to HUVEC cultures at a concentration of 100 μM and for a preincubation period of 24 h. L-Gulonolactone did not affect either calcium-dependent citrulline or cGMP synthesis (Fig. 6). Dehydro-L-ascorbic acid increased ionomycin-stimulated citrulline and cGMP production by 62 and 73%, respectively, and was less effective than L-ascorbic acid (Fig. 6). Ascorbate 2-phosphate potentiated ionomycin-induced citrulline and cGMP formation to a similar extent as L-ascorbic acid, whereas ascorbate 2-sulfate, another derivative with a substituted enediol lactone ring, showed 27 (citrulline) or 20% (cGMP) of the ascorbate effect (Fig. 6).

Effect of Ascorbic Acid on Arginine Uptake into Endothelial Cells—To determine if ascorbic acid affects the transport of the NOS substrate arginine into the cells, HUVEC were incubated in culture medium containing 335 μM L-[14C]arginine (3 mCi/mmol) in the presence or absence of 100 μM ascorbate for 1–24 h. Table I shows that the [14C]arginine uptake was time-dependent but was not modified by ascorbic acid.

Effect of Ascorbic Acid on ecNOS Protein Content in Endothelial Cells—To determine ecNOS expression, cell lysates and subcellular fractions from untreated and ascorbate (100 μM, 24 h)-treated HUVEC were separated by SDS-PAGE and subjected to Western blot analysis using an anti-ecNOS antibody. Fig. 7 shows that the ecNOS was mainly located in the particulate fraction. Differences in ecNOS expression were not seen in whole lysates or in membrane or cytosolic fractions between untreated and ascorbic acid-incubated cells. Western blots from control cells and ascorbate-treated cells were also labeled with a monoclonal antibody against iNOS, but no staining could be detected (not shown).

The expression of ecNOS protein was also measured in permeabilized HUVEC using a flow cytometric technique. The comparison of cellular fluorescence signals with signals from calibration beads revealed about 105 molecules ecNOS per cell. The amount of ecNOS was not changed when HUVEC were incubated with 100 μM ascorbic acid for 24 h as the corrected mean fluorescence intensity was 14.5 ± 2.2 in control cells and 14.6 ± 2.3 in ascorbate-treated cells (n = 10).

Effect of Ascorbic Acid on de Novo Synthesis of ecNOS Protein—To investigate if ascorbic acid leads to an enhanced de novo synthesis of ecNOS protein in HUVEC, lysates from biosynthetically labeled control cells and cells treated with 100 μM ascorbate (6 h preincubation and 3 h coincubation with 35S)methionine medium) were immunoprecipitated with an anti-human ecNOS antibody, resolved by SDS-PAGE, and subjected to autoradiography and subsequent immunostaining. Fig. 8 shows that the 35S-labeled band running at 135 kDa was specific for ecNOS and was not different between control and ascorbate-treated cells suggesting that ecNOS synthesis was not altered by ascorbic acid.

Effect of Ascorbic Acid on Citrulline Formation in Endothelial Cell Lysates—When the calcium-dependent citrulline formation was measured in cell lysates under optimal conditions and with the addition of all known NOS cofactors, the potentiating effect of ascorbate pretreatment (100 μM, 24 h) as seen in intact cells was largely not observed (Fig. 9). This was most probably due to the addition of tetrahydrobiopterin since the
measurement in the presence of all cofactors except tetrahydrobiopterin revealed a 2.5-fold higher citrulline formation in lysates from ascorbate-pretreated cells (Fig. 9). Furthermore, the potentiating effect of ascorbic acid was not observed in reactions where tetrahydrobiopterin was present, but other cofactors (FAD, FMN, and calmodulin) were omitted (results not shown). The citrulline formation in lysates from both untreated and ascorbate-preincubated cells was completely blocked by the addition of 1 mM ML-NAME.

**DISCUSSION**

The present study demonstrates that L-ascorbic acid in physiologically relevant concentrations (28, 29) potentiates agonist-induced endothelial NO synthesis in a dose- and time-dependent fashion. This was shown by concomitant changes of both the formation of the NO co-product citrulline and the accumulation of the NO effector molecule cGMP, although the latter might additionally indicate an increase in biological activity of NO.

The ascorbate effect on NO synthesis was most likely due to an increase of the intracellular ascorbic acid concentration since cell stimulation was performed in the absence of extracellular ascorbate. Under normal culture conditions cells are unlikely to be saturated with ascorbic acid because its concentration in culture media is generally low. Accordingly, an incubation of the cells with ascorbate may lead to an intracellular accumulation of the compound. Indeed, using 100 μM 14C-labeled ascorbate we found an uptake of the compound into endothelial cells which was time-dependent and saturated before 6 h (Fig. 6).
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Fig. 9. Effect of ascorbic acid on citrulline formation in cell lysates. HUVEC were preincubated for 24 h with 100 μM ascorbic acid and subsequently trypsinized and sonicated. Citrulline formation was measured in Hepes buffer (pH 7.2) with 1 mg/ml lysate protein, 20 μM L-[14C]arginine (303 mCi/mmol), 1 μM FAD, 1 μM FMN, 100 μM calmodulin, 2 mM NADPH, and 2.5 mM CaCl2 in the presence (left panel) or absence (right panel, note the different scale units of the x axis) of 2 μM tetrahydrobiopterin (BH4). After 15 min at 37 °C, the mixture was subjected to cation exchange chromatography, and [3H]citrulline was quantified by liquid scintillation counting. The calcium-dependent citrulline formation was expressed in pmol/mg lysate protein (mean ± S.E., n = 6). C, control; AA, ascorbic acid. *p < 0.05 versus untreated control cells.

tween 12 and 24 h. The nature of this uptake is as yet unknown, although the involvement of an active transport mechanism has been demonstrated in endothelial cells (27).

We suggest that the observed rate of ascorbic acid transport into endothelial cells demonstrated in the present study may at least partially account for the time dependence of the ascorbate effect on endothelial NO synthesis. The latter followed a kinetics similar to the ascorbate uptake being about half-maximal after a 6-h incubation of cells with 100 μM ascorbic acid and maximal between 18 and 24 h. Moreover, a saturation of the ascorbate uptake into endothelial cells which might occur between 100 and 200 μM (27) could explain the lack of further NO synthesis potentiation with ascorbic acid concentrations above 100 μM. Interestingly, 100 μM is in the range of plasma levels of healthy individuals (28), and similar concentrations have been found in a recent pharmacokinetic study that described the ascorbic acid concentration as a function of dose in healthy volunteers (29).

The molecular structure of L-ascorbic acid consists of an unsaturated γ-lactone ring with an enediol configuration (–COH=COH) conjugated with a carbonyl group (Fig. 5). Our data suggest that this structure may be essential for the potentiation effect of ascorbate on NO production. L-Gulonolactone, an ascorbic acid precursor molecule, is lacking the enediol configuration and cannot be transformed into ascorbic acid in human cells due to the absence of the enzyme L-gulonolactone oxidase. Accordingly, it did not affect ionomycin-induced citrulline or cGMP synthesis. On the other hand, dehydroascorbic acid which is partially converted back to ascorbate by gluthathione-dependent reactions (30) exerted a partial stimulatory effect, and ascorbate 2-phosphate, known to be hydrolyzed by phosphatases to restore the unsubstituted enediol ring structure (31), was as active as ascorbate itself in potentiating NO synthesis. Similarly, ascorbate 2-sulfate exerted a stimulatory action which was, however, less than 30% of the ascorbate effect. This might be due to a lack of the hydrolyzing enzyme arylsulfatase A (32) and corresponds to studies that have shown that the ascorbic acid activity of ascorbate 2-sulfate was low in humans (33).

Although ascorbic acid clearly enhanced agonist-induced NO production in endothelial cells, it did not induce the expression of eNOS protein. The amount of eNOS recognized with specific monoclonal antibodies in cell lysates or in permeabilized cells was not altered by ascorbate pretreatment. Likewise, ascorbic acid did not induce de novo synthesis of NOS since the 35S-labeled protein band which could be clearly identified as eNOS by immunostaining was not different in untreated and ascorbate-treated cells. The potentiating effect of ascorbic acid on NO synthesis was also not due to a supplementation of the NOS substrate L-arginine because ascorbic acid did not increase the cellular uptake of this amino acid. Since the endothelial NO formation is influenced by the intracellular amount of cofactors such as NADPH, FAD, FMN, tetrahydrobiopterin, and calcium/calmodulin (34), we suggested that ascorbate might act through effects on the availability of these compounds. Indeed, when the NO formation was determined in cell lysates from control cells and ascorbate-pretreated cells under saturated conditions and in the presence of all NOS cofactors, the potentiating effect of ascorbic acid was largely lost. Interestingly, this was most probably due to the addition of tetrahydrobiopterin since a potentiating effect of an ascorbate pretreatment was seen in cell lysates when only tetrahydrobiopterin was omitted from the assay. Moreover, the addition of tetrahydrobiopterin reversed the ascorbate effect in the absence of the cofactors FAD, FMN, or calmodulin, and when tetrahydrobiopterin was omitted, the potentiating effect of an ascorbate pretreatment on citrulline synthesis could be seen regardless whether FAD, FMN, and calmodulin were added to the cell lysates (data not shown). From these results we can speculate that ascorbic acid may at least partially act through an effect on the availability of tetrahydrobiopterin or enhance the affinity of this cofactor toward eNOS. An increase of intracellular tetrahydrobiopterin levels has already been shown to stimulate the constitutive NOS activity suggesting that the eNOS is incompletely saturated with this cofactor (25, 36). Tetrahydrobiopterin has been suggested to stabilize the active homodimeric NOS (37) and probably acts as both a redox-active cofactor of L-arginine oxidation and an allosteric effector of the enzyme protein (38). The possibility that ascorbic acid may enhance the apparent affinity of tetrahydrobiopterin for the neuronal NOS isoform has also been suggested (39).

So far, protective vascular effects of ascorbic acid have been attributed to its radical scavenging properties which may lead to a protection of NO from inactivation by superoxide anion and to an increase of NO bioavailability. These mechanisms may be important in vivo and may explain the improvement of endothelium-dependent vasodilatation in cardiovascular patients by an acute ascorbic acid application (18–23). The results presented in this study describe a new mechanism for vascular protection by ascorbic acid which may be effective when plasma levels of ascorbic acid supply saturated intracellular ascorbate concentrations. We suggest that tissue saturation with ascorbic acid provides the optimal reaction conditions for adequate NO synthesis in endothelial cells and that a decrease in the cellular ascorbic acid content may support the development of endothelial dysfunction. Future clinical studies investigating ascorbate effects on endothelial functions should thus include parameters that evaluate NO formation in vivo.

In summary, this study demonstrates that L-ascorbic acid potentiates agonist-induced NO formation in cultured endothelial cells in a dose-dependent fashion. The effect was time-dependent, related to an increase of intracellular ascorbate levels, and saturated within physiologically relevant concentrations. Ascorbic acid did not induce the expression of eNOS protein and may at least partially act through an effect on the availability or affinity of tetrahydrobiopterin for eNOS. Further studies are in progress to clarify these suggested mechanisms. The findings presented in this study suggest that NO formation in endothelial cells depends on tissue ascorbate levels and that
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tissue saturation with ascorbic acid may add to the strategies for an improvement of endothelial vasodilator function in humans.

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