Molecular Human Reproduction Vol.8, No.3 pp. 271–280, 2002

Chemical stabilization of tetrahydrobiopterin by L-ascorbic acid: contribution to placental endothelial nitric oxide synthase activity

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The aim of this study was to characterize the mechanism of the chemical interaction between L-ascorbic acid (ASC) and tetrahydrobiopterin (BH_4) *in vitro* and to examine its effect on the activity of endothelial nitric oxide synthase (eNOS) in first trimester human placentae. At room temperature, in Tris–HCl buffer (pH 7.4), both ASC and BH_4 were readily oxidized by dissolved O_2 or H_2O_2. BH_4 was more sensitive to auto-oxidation, while ASC was more susceptible to oxidation by H_2O_2. Addition of 36 μmol/l BH_4 to 143 μmol/l ASC increased the initial rate of ASC oxidation 3.2-fold in a catalase-sensitive manner, indicating that enhanced ASC oxidation is partly due to the formation of H_2O_2. In the presence of catalase, BH_4 still stimulated 1.9-fold the initial rate of ASC oxidation, suggesting that another auto-oxidation product of BH_4, most probably quininoid-BH_2 (qBH_2), could also stimulate ASC oxidation while itself being reduced back to BH_4. ASC prevented the auto-oxidation of BH_4 in a concentration-dependent fashion, with 3 mmol/l ASC providing an almost complete stabilization of 25 μmol/l BH_4. Importantly, basal eNOS activity in placental microsomes was stimulated 2.5-fold by 0.5 μmol/l BH_4, and 0.5 mmol/l ASC enhanced the BH_4-stimulation 1.4-fold, with a smaller effect on basal eNOS activity. Taken together, the findings support the notion that the stabilizing action of ASC on BH_4 is related to the ASC-mediated reductive reversal of the auto-oxidation process of BH_4. Moreover, we demonstrated that concentrations of ASC present in the placenta as a common vitamin C supply are sufficient to protect cellular free BH_4 and may contribute to the stimulation of placental eNOS activity.

Key words: ascorbate/auto-oxidation/eNOS/human placenta/tetrahydrobiopterin

Introduction

Tetrahydrobiopterin (BH_4, 6R-L-erythro-5,6,7,8-tetrahydrobiopterin) is an indispensable co-factor for the activity of nitric oxide synthase (NOS) enzymes. BH_4 is an unstable compound; it undergoes auto-oxidation in aqueous solutions at pH 7.4 to form 7,8-dihydrobiopterin (BH_2), with tetrahydrobiopterin 4a-hydroperoxid and quininoid dihydrobiopterin (qBH_2) as possible intermediates (Thöny *et al*., 2000).

A close link between cellular BH_4 concentrations and NO synthesis has been described for a number of different cell types including endothelial cells and placental syncytiotrophoblasts (Schmidt *et al*., 1992; Conrad *et al*., 1993; Myatt *et al*., 1993a; Werner-Felmayer *et al*., 1993; Buttery *et al*., 1994; Rosenkranz-Weiss *et al*., 1994; Kukor *et al*., 1996, 2000; Tóth *et al*., 1997). Moreover, BH_4 supplementation has been demonstrated to restore or improve endothelium-dependent vasodilation in several pathological states including atherosclerosis (Tiefenbacher *et al*., 2000), reperfusion injury following transient coronary occlusion (Tiefenbacher *et al*., 1996), diabetes mellitus (Pieper, 1997), hypercholesterolaemia (Stroes *et al*., 1997) and vascular dysfunctions of chronic smokers (Heitzer *et al*., 2000; Ueda *et al*., 2000).

Ascorbic acid, one of the most important water-soluble physiological antioxidants, has also been shown to improve endothelial vasodilating functions in atherosclerosis (Levine *et al*., 1996; Ness *et al*., 1996a; Gokce *et al*., 1999; Frei, 1999); diabetes mellitus (Ting *et al*., 1996; Timimi *et al*., 1998) and hypercholesterolaemia (Ting *et al*., 1997; Jeserich *et al*., 1999) and to alleviate the endothelial dysfunction of chronic smokers (Heitzer *et al*., 1996). In addition, a number of studies have concluded or suggested that a low plasma ascorbic acid level (taken as an indicator of vitamin C deficiency) is a risk factor for coronary heart disease (Riemersma *et al*., 1991; Enstrom *et al*., 1992; Gey *et al*., 1993; Ness *et al*., 1996b; Nyyssönen *et al*., 1997; Vita *et al*., 1998) and stroke (Gey *et al*., 1993; Gale *et al*., 1995). Of

particular interest are the observations that ascorbic acid may contribute to the lowering of blood pressure (Ness *et al*., 1996c, 1997; Frei, 1999).

Low ascorbic acid levels have been reported by several laboratories (Mikhail *et al*., 1994; Hubel *et al*., 1997; Sagol *et al*., 1999; Kharb, 2000) in pre-eclampsia. This finding is not surprising since increased oxidative stress appears to be a cardinal factor in the pathogenesis of this hypertensive disorder (Davidge, 1998; Dekker and Sibai, 1998; Hubel, 1999). Combined antioxidant therapy protocols including high doses of vitamin C and vitamin E have led to some beneficial effects in pre-eclamptic patients (Gulmezoglu *et al*., 1997; Chappell *et al*., 1999).

An explanation for the related vascular effects of BH_4 and ascorbic acid has emerged from the observations that ascorbic acid potentiates NO synthesis in a BH_4-dependent manner in endothelial cells obtained from human umbilical veins and coronary arteries (Heller *et al*., 1999), or from porcine aorta (Huang *et al*., 2000). Similar effects have been observed *in vitro* using purified recombinant bovine eNOS (Huang *et al*., 2000). Further studies have revealed that treatment of endothelial cells with physiological concentrations of ascorbic acid leads to an increase in intracellular BH_4 levels and this effect can be ascribed solely to a chemical stabilization of this cofactor (Baker *et al*., 2001; Heller *et al*., 2001).

In this study, first we examined how ascorbic acid and BH_4, which are two auto-oxidizable compounds, interact with each other and how this interaction results in chemical protection of BH_4. We conclude that O_2 reacts with BH_4 more avidly than with ascorbic acid, and ascorbic acid exerts a direct reducing effect on the oxidation product of BH_4, presumably on qBH_2. Ascorbic acid also efficiently removes H_2O_2, the main product of BH_4 auto-oxidation, and a powerful oxidant. Furthermore, we provide evidence that ascorbic acid potentiates the stimulatory effect of BH_4 on placental eNOS activity *in vitro*. Finally, determinations of ascorbic acid concentrations in placental tissues obtained from different pregnancies confirm that the BH_4-stabilizing chemical effect of ascorbic acid might be functional under physiological conditions.

Materials and methods

Materials

L-[U-^{14}C]arginine (298 mCi/mmol; 11 GBq/mmol) was obtained from ICN (Costa Mesa, CA, USA). L-ascorbic acid, Tris base, EDTA and hydrogen peroxide (H_2O_2) were purchased from REANAL (Budapest, Hungary). Tetrahydrobiopterin, biopterin, crystalline catalase from bovine liver, L-N^G-nitroarginine methyl-ester (NAME), ethyleneglycol-bis(β-aminoethylether)-N-tetra-acetate (EGTA), NADPH, Dowex 50X8-400, dithiotreitol (DTT), citrulline, calmodulin, leupeptin, soybean trypsin inhibitor and aprotinin were from Sigma Chemical Co. (Budapest, Hungary). Phenylmethylsulphonylfluoride (PMSF) and HEPES were from Calbiochem (La Jolla, CA, USA). Reagents were prepared with double-distilled deionized water. L-ascorbic acid and BH_4 stock solutions were prepared in 0.1 mmol/l HCl and aliquots were stored at –30°C. Ascorbic acid was diluted prior to experiments with 50 mmol/l Tris–HCl (pH 7.4). Concentrations of H_2O_2 stock solutions were determined by permanganometric titration.

Measurement of ascorbic acid oxidation

To determine the rate of auto-oxidation, ascorbic acid (100 or 143 µmol/l final concentration) was dissolved in 3.5 ml of 50 mmol/l Tris–HCl (pH 7.4) or 50 mmol/l Tris–HCl, 50 µmol/l or 0.5 mmol/l EDTA (pH 7.4) buffer. A decrease in optical absorbance at 265 nm was monitored in the presence or absence of 25 or 36 µmol/l BH_4 or catalase (8.6, 17.2 or 27.1 µg/ml) or H_2O_2 (0.06, 0.30 or 1.20 mmol/l) at 22°C, using a Hitachi U-2001 double beam spectrophotometer. The concentration of oxidized ascorbic acid was calculated from the decrease in absorbance at 265 nm using the molar extinction coefficient of 16 500 for ascorbic acid (Davies *et al*., 1991).

Measurement of BH_4 oxidation

Oxidation of BH_4 was followed by monitoring the decrease of absorbance at 295 nm at 22°C. The concentration of oxidized BH_4 was calculated using a molar extinction coefficient of 5500 (M.Tóth, unpublished data). In the presence of ascorbic acid, auto-oxidation of BH_4 was studied by measuring the absorbance decrease at 305 nm where the interference by absorption of ascorbic acid was negligible and concentration changes were calculated on a percentage basis. Incubations were initiated by adding an appropriate volume of BH_4 to a freshly diluted ascorbic acid solution or incubation buffer to reach a final volume of 3.5 ml. Where indicated, H_2O_2 (0.06, 0.3 and 1.2 mmol/l) or catalase (8.6 or 17.2 µg/ml) were included in the incubation mixtures.

Tissue, homogenization and fractionation

First trimester human placentae from legal instrumental termination of 9–11 week old pregnancies were obtained from the 2nd Department of Obstetrics and Gynecology, Semmelweis University, Budapest. Use of the placentae for these experiments has been approved by the Ethics Committee of the clinical department and informed consent was given by each patient. Minced villous placentae were homogenized in two volumes of ice-cold homogenizing solution containing 0.3 mol/l sucrose, 40 mmol/l HEPES-Na (pH 7.4), 0.1 mmol/l EDTA, 1 mmol/l DTT, 1 mmol/l PMSF, 10 µg/ml leupeptin, 10 µg/ml soybean trypsin inhibitor and 0.2 µg/ml aprotinin, using an UltraTurrax apparatus (IKA Werk, Staufen, Germany) at the three-quarter setting for 30 s. The homogenate was filtered through a nylon mesh and subjected to centrifugation at 15 000 *g* for 30 min in a Beckman J-21 centrifuge. The supernatant was then centrifuged at 100 000 *g* for 60 min in a Beckman L2-65B ultracentrifuge to obtain the microsomal pellet. The pellet was rinsed three times with DTT-free homogenizing solution and suspended in a small volume of the same solution. Four sets of microsomes were prepared from placental tissues obtained from each of three first-trimester human pregnancies and stored at –80°C until measurement of enzyme activity.

Measurement of NO synthase activity

NOS activity was determined by measuring the rate of conversion of [^{14}C]arginine into [^{14}C]citrulline. 100 µl tissue extract containing 1.4–1.8 mg protein was incubated with 0.15 µCi [^{14}C]arginine (2 µmol/l final concentration), 0.4 mmol/l NADPH, 1 mmol/l citrulline, 1 mmol/l $MgCl_2$, 1 mmol/l $CaCl_2$, 0.1 mmol/l EGTA, 3 IU calmodulin, 20 mmol/l HEPES-Na (pH 7.4) and BH_4 and ascorbic acid as indicated, in 250 µl final volume for 15 min at 37°C. Incubations were run in duplicates. Control incubates contained 1 mmol/l EGTA without Ca^{2+} added. In separate control incubations, 1 mmol/l EGTA and 1 mmol/l L-NAME were present in the absence of exogenous Ca^{2+} in order to measure Ca^{2+}-independent citrulline formation which was negligible in these experiments. [^{14}C]citrulline was separated from [^{14}C]arginine on small columns of Dowex 50X8-400 cation exchange resin and radioactivity was measured using a

Packard Tri-Carb 2100 TR liquid scintillation analyzer. Measured radioactivities were corrected for the mean of the radioactivity values of controls and eNOS activity was calculated as disintegrations/min (d.p.m.) of incubation per mg of protein. Finally, from the duplicate results the mean value ± SD from the mean was computed. Experiments were repeated three times with different microsomal preparations, the results were normalized to the mean of activity values obtained in the absence of BH_4 and ASC and subjected to statistical analysis.

Determination of placental concentration of ascorbate

For determination of total ascorbic acid (i.e. ascorbic + dehydroascorbic acids), a published procedure (Denson and Bowers, 1961) was used. The method measures ascorbic acid in TCA extracts after oxidation by Cu^{2+} ions and conjugation with 2,4-dinitrophenylhydrazine. Triplicate placental pieces (1 g each), obtained at term from normal pregnancies or from first trimester pregnancies after legal interruption, were homogenized in 10% TCA with an all-glass Potter–Elvehjem homogenizer and the TCA-soluble fraction was collected by centrifugation. The TCA concentration of the extract was adjusted to 5% and asorbic acid was measured from duplicate aliquots. A calibration line was prepared using pure ascorbic acid dissolved in 5% TCA.

Ascorbic acid was determined using the dipyridyl method (Omaye *et al*., 1979). The procedure is based on the quantitative oxidation of ascorbic acid by Fe^{3+} and the subsequent conversion of Fe^{2+} with dipyridyl into an orange–yellow chelate complex. Placental pieces (1 g each) were homogenized in 4 ml ice-cold 0.9% NaCl, 100 μmol/l EDTA solution using the UltraTurrax apparatus mentioned above, the homogenate was briefly centrifuged in the cold and a 1.0 ml clear aliquot from the supernatant was added to 4 ml 5% TCA, 50 μmol/l EDTA. After centrifugation, ascorbic acid determinations were made from aliquots of the clear supernatant. Calibration line was prepared from ascorbic acid dissolved in 5% TCA, 50 μmol/l EDTA solution.

Determination of protein

Protein was measured by a published method (Lowry *et al*., 1951) using bovine serum albumin as standard.

Statistical analysis

For statistical evaluation of data, one-way analysis of variance followed by Bonferroni's *t*-test or Wilcoxon's unpaired *t*-test was used. $P < 0.05$ was considered to be statistically significant. For comparison of reaction rates of ascorbic acid oxidation, the initial rate was used. The reaction rate was regarded initial as far as it was directly proportional to the reaction time.

Results

Effect of auto-oxidation on the absorption spectra of ascorbic acid and BH_4

The UV spectrum of ascorbic acid at pH 7.4 exhibited a high absorption peak with a maximum at 265 nm (Figure 1A). Oxidation of ascorbic acid led to a loss of this absorption band (data not shown), therefore, ascorbic acid oxidation was determined by monitoring the optical density at 265 nm.

The UV absorption spectra of BH_4 before and after incubation in Tris–HCl (pH 7.4) at room temperature for 40 min were studied (Figure 1B). The spectrum of BH_4 exhibited characteristic changes as BH_4 was rapidly auto-oxidized. Apparently, complete oxidation of BH_4 to biopterin did not occur, because the absorption spectrum of biopterin is different from the spectrum obtained for BH_4 after incubation. The decrease in absorption of BH_4 at 295 nm during oxidation offered a convenient way to monitor this process.

Inclusion of 25 μmol/l BH_4 in an ascorbic acid solution of 100 μmol/l resulted in only a slight absorption elevation at ~300 nm (Figure 1A). This elevation was due to the absorption of BH_4 and it allowed selective monitoring of BH_4 auto-oxidation at 305 nm without substantial interference by light absorption of ascorbic acid. At the same time, oxidation of ascorbic acid could still be quantified by measuring the decrease in absorption at 265 nm, since BH_4 and BH_2 show a similar quench at 265 nm (Figure 1B) and redox changes of BH_4 do not interfere at this wavelength.

Oxidation of ascorbic acid and BH_4 by O_2 or H_2O_2 dissolved in the medium

Ascorbic acid (100 μmol/l final concentration) underwent fairly rapid auto-oxidation at pH 7.4 with a half-time ($T_{1/2}$) of 35 min and the rate of this change was only slightly influenced by the presence of 25 μmol/l BH_4 (Figure 2A). On the other hand, inclusion of 50 μmol/l EDTA in the buffered solution markedly reduced the rate of auto-oxidation ($T_{1/2} > 120$ min), indicating that metal contaminants present in the buffer enhance auto-oxidation of ascorbic acid. Under these conditions, BH_4 exerted a 1.8-fold stimulatory effect on ascorbic acid oxidation (Figure 2B).

The reactivity of ascorbic acid and BH_4 with H_2O_2 was also studied. Oxidation of ascorbic acid (36 μmol/l) was accelerated markedly by low concentrations of H_2O_2 (Figure 3A) while the effect of H_2O_2 on BH_4 (36 μmol/l) was much smaller (Figure 3B). For instance, 60 μmol/l H_2O_2 resulted in 3-fold and 1.2-fold acceleration in the initial rate of ascorbic acid and BH_4 auto-oxidation respectively. Taken together, Figures 2 and 3 clearly demonstrate that: (i) BH_4 reacts faster with O_2 than does ascorbic acid (i.e. BH_4 is more susceptible to auto-oxidation than is ascorbic acid); (ii) the opposite holds true for H_2O_2: BH_4 oxidation is only slightly increased, whereas ascorbic acid oxidation is significantly enhanced, in response to H_2O_2.

In order to compare the contribution of H_2O_2, formed during auto-oxidation of both ascorbic acid and BH_4, to the oxidation of these compounds, the effect of catalase on the initial rate of their auto-oxidation was also studied. The oxidation rate of ascorbic acid was inhibited by 8.6 and 17.2 μg/ml catalase at 38.7 and 51.9% respectively, in the presence of 50 μmol/l EDTA (Figure 4A). This value definitely exceeded the 25% catalase-induced decrease in the initial rate of BH_4 auto-oxidation measured under the same conditions (Figure 4B). Evidently, ascorbic acid is more sensitive than BH_4 to the H_2O_2 generated during the auto-oxidation of these compounds.

Ascorbic acid protects BH_4 against oxidation

In order to gain insight into the redox mechanisms between ascorbic acid and BH_4, the effect of catalase on the spontaneous and the BH_4-promoted ascorbic acid oxidation was studied using the standard experimental conditions (using 100 μmol/l

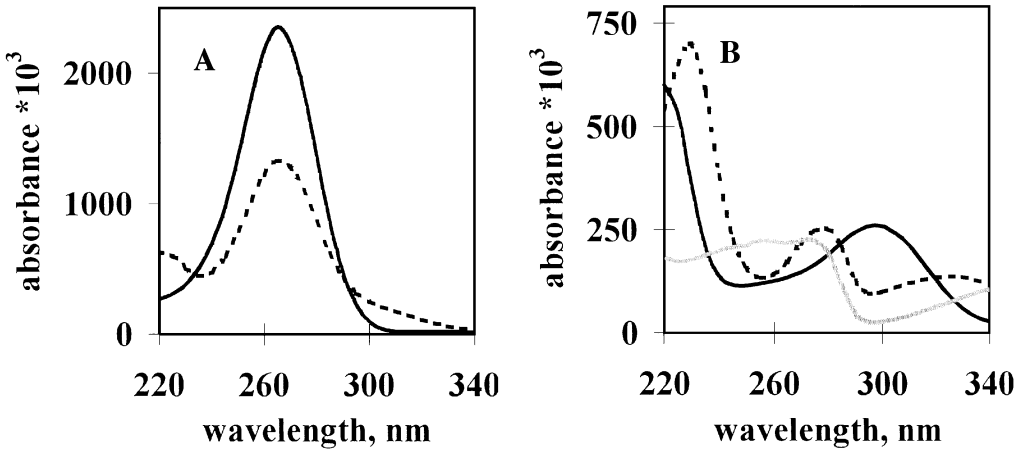

Figure 1. Ultraviolet absorption spectra of ascorbic acid (ASC), tetrahydrobiopterin (BH_4) and biopterin. (**A**) Ultraviolet absorption spectrum of ascorbic acid (ASC) in the presence or absence of BH_4: UV absorption of ASC dissolved in 50 mmol/l Tris–HCl, pH 7.4 at 200 µmol/l final concentration was monitored between 220 and 340 nm using a Hitachi U-2001 double-beam, automatic spectrophotometer (solid line). Absorption spectrum of the mixture of 100 µmol/l ASC and 25 µmol/l BH_4 is shown by the dashed line. (**B**) Ultraviolet absorption spectra of tetrahydrobiopterin (BH_4) and biopterin: UV absorption of BH_4 dissolved in 50 mmol/l Tris–HCl, pH 7.4, at 25 µmol/l final concentration was monitored between 220 and 340 nm before (solid line) and after (dashed line) incubation at 22°C for 40 min. The UV spectrum of biopterin is shown by the grey line.

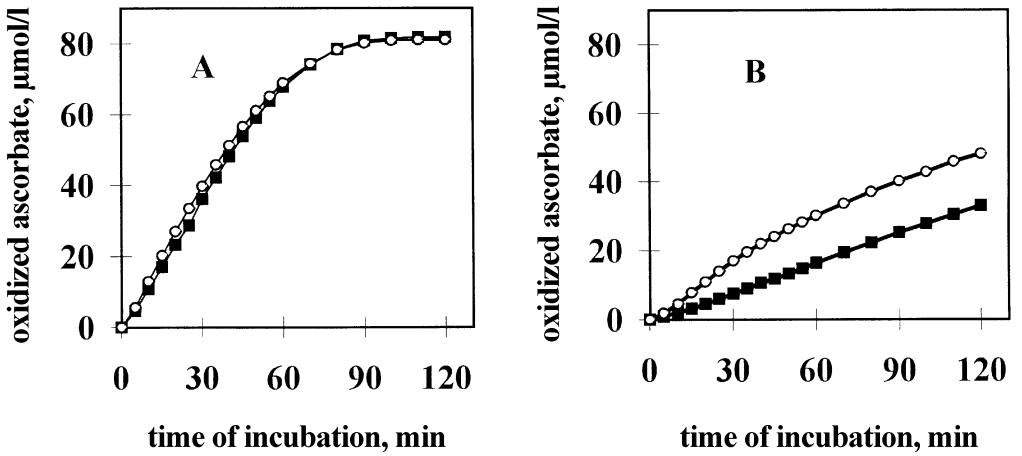

Figure 2. Effect of tetrahydrobiopterin (BH_4) on the oxidation of ascorbic acid (ASC) in the absence (**A**) or presence (**B**) of EDTA. ASC (100 µmol/l) was incubated in 3.5 ml 50 mmol/l Tris–HCl, pH 7.4 (**A**) or 50 mmol/l Tris–HCl, pH 7.4, 50 µmol/l EDTA (**B**) solution for the time periods indicated, in the presence (open circles) or absence (solid squares) of 25 µmol/l tetrahydrobiopterin (BH_4). Oxidation of ASC was monitored by measuring the decrease in optical density at 265 nm. Incubations were performed in triplicates and mean values ± SD are presented. In most cases, SD values fall within the size of the symbol. Data from one representative experiment out of three giving similar results are presented.

ascorbic acid, 25 µmol/l BH_4 and 50 µmol/l EDTA). BH_4 increased 1.8-fold the initial rate of ascorbic acid oxidation. In the presence of 8.6 µg/ml catalase, the rate of BH_4-dependent ascorbic acid oxidation decreased markedly, and some attenuation of the BH_4-independent auto-oxidation of ascorbic acid also occurred (data not shown). However, in the presence of 8.6 µg/ml catalase, the rate of ascorbic acid oxidation measured in the presence of BH_4 was 1.25-fold the rate of ascorbic acid oxidation determined in the absence of BH_4. This definite rate difference did not change in the presence of a 2-fold concentration (17.2 µg/ml) of catalase indicating that incomplete removal of H_2O_2 was not responsible for the observed residual oxidation of ascorbic acid (data not shown). These observations seemed to reflect a crucial H_2O_2-independent interaction between BH_4 auto-oxidation and ascorbic acid oxidation. In order to support the validity of this finding, a series of further measurements was performed. With the aim to increase the effect of BH_4 on ascorbate oxidation in the presence of catalase, in these experiments the concentrations of ascorbic acid, BH_4 and EDTA in the incubation medium were enhanced to 143 and 36 µmol and 0.5 mmol/l respectively. In addition, four series of experiments with duplicate incubations each were performed and the results were subjected to statistical analysis. The results indicated an extremely significant ~2-fold BH_4-dependent increase in the initial rate of ascorbic acid oxidation in the presence of 27.1 µg/ml catalase (Figure 5). This oxidation could not be caused by incomplete removal of H_2O_2, because another series of control experiments clearly demonstrated that concentrations of catalase in the range of 17.2–27.1 µg/ml (smaller concentra-

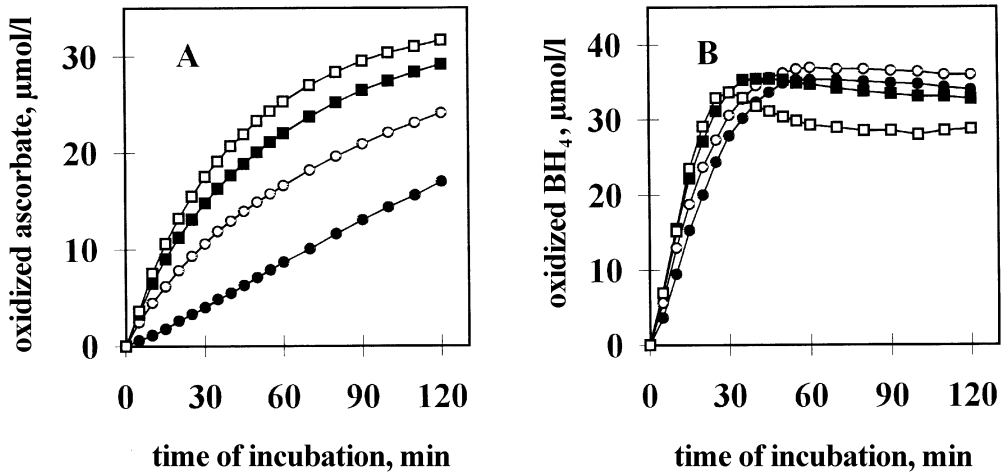

Figure 3. Effect of various concentrations of H_2O_2 on the oxidation of ascorbic acid (ASC) (**A**) and tetrahydrobiopterin (BH_4) (**B**). ASC or BH_4 (36 µmol/l each) was incubated without H_2O_2 (control, solid circles) or with each of three different concentrations of H_2O_2: 0.06 mmol/l (open circles), 0.30 mmol/l (solid squares) and 1.20 mmol/l (open squares) in 50 mmol/l Tris–HCl, pH 7.4, 50 µmol/l EDTA at 22°C for the time periods indicated. Oxidation of ASC and BH_4 was followed by the decrease of optical density at 265 and 295 nm respectively. Data from one representative experiment out of three giving similar results are presented.

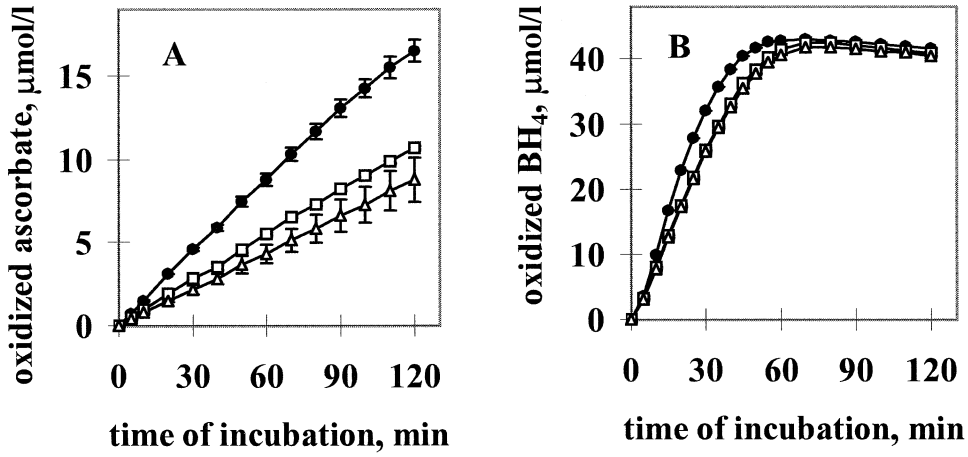

Figure 4. Effect of catalase on the auto-oxidation of ascorbic acid (ASC) (**A**) and tetrahydrobiopterin (BH_4) (**B**). ASC or BH_4 (36 µmol/l each) was incubated in 50 µmol/l Tris–HCl buffer, pH 7.4, 50 µmol/l EDTA in the absence (control, solid circles) or presence of 8.6 µg/ml (open squares) or 17.2 µg/ml (open triangles) catalase. Oxidation of ASC and BH_4 was followed by the decrease of optical density at 265 and 295 nm respectively. Each point is a mean value of duplicate incubations. Data from one representative experiment out of three giving similar results are presented.

tions were not studied) completely prevent the oxidation of ascorbate by 60 µmol/l H_2O_2 (data not shown). These results indicated that H_2O_2 released from the auto-oxidation of BH_4 is an important, but not the only, factor leading to oxidation of ascorbic acid. Since in the presence of catalase BH_4 still accelerated up to 2-fold the oxidation rate of ascorbic acid, we concluded that beside H_2O_2, BH_2 (or rather the quininoid-BH_2 intermediate of BH_4 auto-oxidation) was also able to oxidize ascorbic acid. Because in the absence of H_2O_2 only BH_2 (or qBH_2) is present in the system as a redox reaction partner for ascorbic acid, it is reasonable to suggest that ascorbic acid reduced this compound to form BH_4.

In order to demonstrate the protective effect of ascorbic acid on net BH_4 oxidation, BH_4 stability was measured in the presence of various concentrations of ascorbic acid (Figure 6). In these measurements, EDTA was not included in the incubated mixtures and ascorbic acid solutions at the tested concentrations were used as photometric blanks (i.e. test solutions and blank solutions contained the same concentration of ascorbic acid). Since in the absence of EDTA there is only negligible difference between the rates of oxidation of ascorbic acid measured either in the presence or absence of 25 µmol/l BH_4 (Figure 2A), possible interference by optical absorption of ascorbic acid with the BH_4 absorption measured at 305 nm was largely eliminated. As shown by Figure 6, ascorbic acid inhibits BH_4 auto-oxidation in a concentration-dependent fashion with 3 mmol/l ascorbic acid providing an almost perfect stabilization of 25 µmol/l BH_4.

Ascorbic acid stimulates microsomal eNOS activity of the human placenta

Keeping in mind the definite protective action of ascorbic acid on BH_4 *in vitro*, the protective effect of ascorbic acid on eNOS activity of placental microsomes was investigated. In these

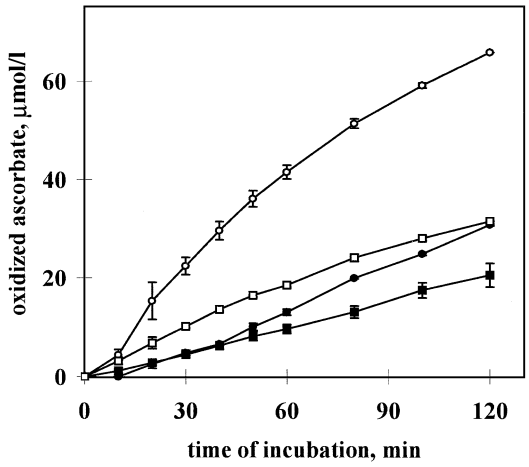

Figure 5. Effect of catalase on the tetrahydrobiopterin (BH_4)-dependent and BH_4-independent oxidation of ascorbic acid (ASC). Oxidation of ASC (143 µmol/l) was monitored at 265 nm using 50 mmol/l Tris–HCl, pH 7.4, 0.5 mmol/l EDTA mixture as solvent. Auto-oxidation of ASC in the absence of BH_4 and catalase served as control (solid circles). Other incubates contained (in addition to ASC): 36 µmol/l BH_4 (open circles), 27.1 µg/ml catalase (solid squares) and 36 µmol/l BH_4 plus 27.1 µg/ml catalase (open squares). Results are from four experiments with duplicate incubations each and mean values ± SEM ($n = 4$) are presented. SEM values are indicated by error bars; where the bar is not visible it is within the size of the symbol. Statistical analysis (ANOVA followed by Bonferroni's *t*-test) on the basis of the initial rates of ascorbate oxidation: +catalase, $+BH_4$ versus +catalase, $–BH_4$: $P < 0.001$; +catalase, $+BH_4$ versus –catalase, $–BH_4$: $P < 0.01$; +catalase, $–BH_4$ versus –catalase, $–BH_4$: $P > 0.05$ (not significant); –catalase, $+BH_4$ versus the other three: $P < 0.001$.

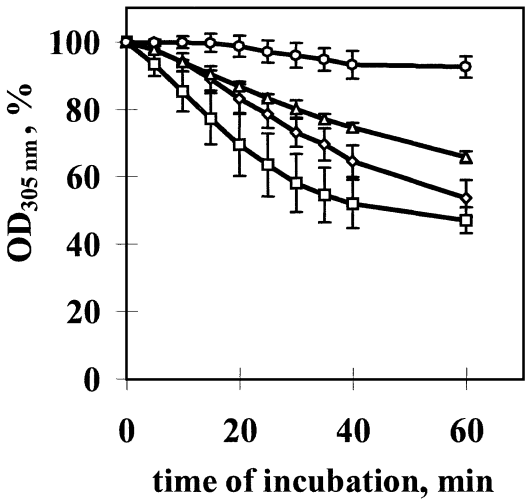

Figure 6. Effect of different concentrations of ascorbic acid (ASC) on the auto-oxidation of tetrahydrobiopterin (BH_4). BH_4 (25 µmol/l) was incubated at 22°C in the absence (squares) or in the presence of 0.5 mmol/l (diamonds) or 1.5 mmol/l (triangles) or 3 mmol/l (circles) final concentration of ASC in 50 mmol/l Tris–HCl, pH 7.4 for the time periods indicated and the optical density at 305 nm was recorded. ASC concentrations of blank solutions were exactly identical to that of the test solutions. Mean values ± SD ($n = 4$–6) are presented. Statistical analysis (ANOVA followed by Bonferroni's *t*-test) on the basis of the initial rates of decomposition: 0.5 mmol/l ASC versus control or 1.5 mmol/l ASC: $P > 0.05$ (not significant); 1.5 mmol/l ASC versus control: $P < 0.01$; 3.0 mmol/l ASC versus control: $P < 0.001$; 3.0 mmol/l ASC versus 0.5 or 1.5 mmol/l ASC: $P < 0.01$.

experiments the otherwise routinely used DTT was omitted, since the strong reducing power of this non-physiological dithiol would mask the protective effect of ascorbic acid. In addition, EGTA (100 µmol/l final concentration) was included in the incubation mixtures to protect ascorbic acid against metal-catalysed auto-oxidation. Physiological BH_4 concentrations stimulated eNOS activity 2.5-fold, and ascorbic acid afforded an additional 1.4-fold increase in the BH_4-stimulatable eNOS activity (Figure 7). The activity increase was maximal at 500 µmol/l ascorbic acid (Figure 7A) and at 200–500 nmol/l BH_4 (Figure 7B) concentrations. However, a significant increase of enzyme activity was observed already at 100 µmol/l ascorbic acid concentration (Figure 7A).

Ascorbic acid concentrations in the human placenta

First trimester and term human placentae contained 117.4 ± 12.5 µmol/kg (mean ± SD, $n = 3$ placentae) and 254.5 ± 85.2 µmol/kg tissue (mean ± SEM, $n = 7$ placentae) ascorbic acid when measured as the dehydroascorbic acid form. Determination of ascorbic acid on the basis of its reducing capacity (314.3 ± 47.5 µmol/kg, mean ± SEM, $n = 7$) did not show a statistically significant difference ($P = 0.5513$). This finding indicates that most of the ascorbic acid is present in reduced form in placental tissues and in sufficient concentrations to exert a protective effect on auto-oxidative BH_4 inactivation.

Discussion

NO is produced during human pregnancy by type III (endothelial) NOS (Conrad *et al*., 1993; Garvey *et al*., 1994; Gude *et al*., 1994; Kukor and Tóth, 1994; Tóth *et al*., 1995) in syncytiotrophoblasts and in the endothelial cells of the umbilical and villous blood vessels (Conrad *et al*., 1993; Myatt *et al*., 1993a,b; Buttery *et al*., 1994; Eis *et al*., 1995). Adequate NO production is considered to be important in the maintenance of feto-placental and materno-placental perfusion and in the adaptation of maternal circulation by vasodilation and blood pressure decrease to the expanded blood volume during pregnancy (Gude *et al*., 1990; Myatt *et al*., 1991, 1992; Chaudhuri *et al*., 1993; Hull *et al*., 1994; Rutherford *et al*., 1995). Our previous results have indicated that eNOS of human placenta is not saturated with BH_4, therefore elevation of BH_4 levels can stimulate enzyme activity and NO production in placental tissues (Kukor *et al*., 1996, 2000; Sahin-Tóth *et al*., 1997; Tóth *et al*., 1997, 1998). eNOS is active as a dimer and, similarly to neuronal NOS (Gorren *et al*., 1996; Riethmüller *et al*., 1999) and inducible NOS (Mayer *et al*., 1997), one of its subunits appears to bind BH_4 tightly, while the affinity of the second subunit to BH_4 is much lower and the binding concentrations fall within the tissue concentration range of BH_4 (Tóth *et al*., 1998; Kukor *et al*., 2000). Consequently, variations in cellular BH_4 concentrations can regulate NO production via the second subunit of eNOS. Decreased availability of BH_4 may cause uncoupling of oxygen reduction and arginine oxidation by eNOS and may lead to generation of superoxide anions and subsequently H_2O_2 (Stroes *et al*., 1998; Vásquez-Vivar *et al*., 1998; Xia *et al*., 1998; Leber *et al*.,

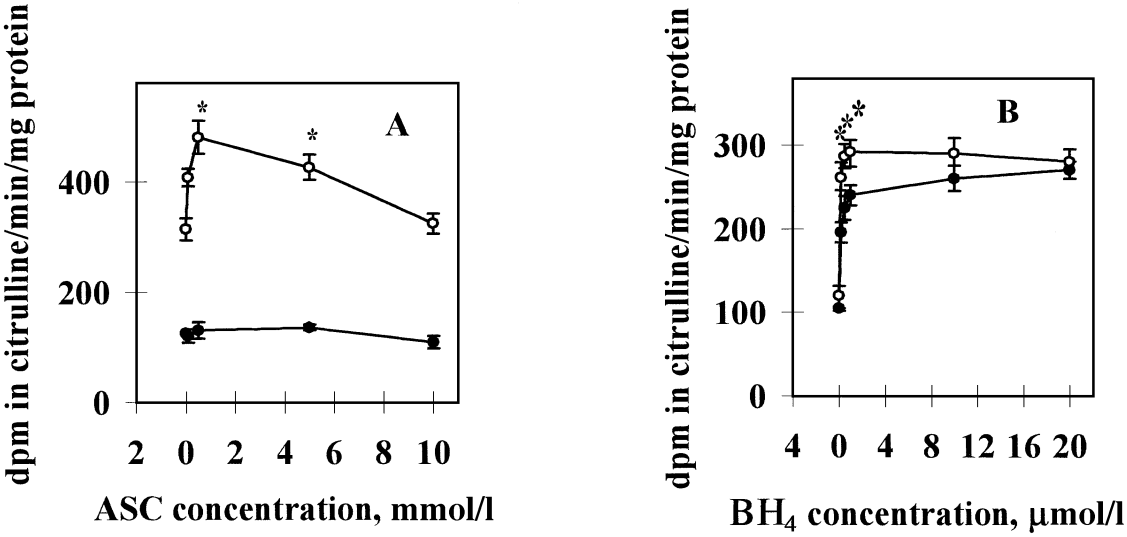

Figure 7. Effect of ascorbic acid (ASC) on the basal and tetrahydrobiopterin (BH_4)-stimulated endothelial nitric oxide synthase (eNOS) activities of placental microsomes. (**A**) Effect of various concentrations of ASC at constant BH_4 concentration. Microsomal suspensions (1.4 mg protein) were incubated in duplicates using the standard reaction mixture containing various concentrations of ASC [0.0 (control), 0.1, 0.5, 5.0 and 10.0 mmol/l respectively] in a final volume of 250 µl for 15 min at 37°C in the absence (solid circles) or presence (open circles) of 0.5 µmol/l BH_4. (**B**) Effect of various concentrations of BH_4 at constant ASC concentration. Microsomal suspensions (1.8 mg protein) were incubated in duplicates with various concentrations of BH_4 [0.0 (control), 0.2, 0.5, 1.0, 10.0 and 20.0 µmol/l respectively] in the absence (control, solid circles) or presence (open circles) of 5.0 mmol/l ASC using the standard reaction mixture, for 15 min at 37°C. Mean values from four experiments ($n = 4$) with duplicate determinations each ± SEM (shown by error bars) are presented. Asterisks indicate significant differences ($P < 0.05$) from activities obtained with microsomes incubated with BH_4 in the absence of ASC (**A**; one-way ANOVA followed by Bonferroni's *t*-test) or between microsomes incubated in the absence and presence of ASC (**B**; Wilcoxon's unpaired *t*-test).

1999). Thus BH_4 deficiency may cause both impaired NO formation and increased production of oxygen radicals. Moreover, NO and superoxide can combine with each other to form peroxynitrite ($ONOO^-$), an aggressive oxidant (Beckman and Koppenol, 1996; Cosentino and Lüscher, 1998). Coupled with NO inactivation, the formation of peroxynitrite may constitute a serious risk factor for hypertension and atherosclerosis and, in the case of pregnancy, for placental ischaemia, a putative cause of pre-eclampsia (Davidge, 1998; Dekker and Sibai, 1998; Hubel, 1999; Lowe, 2000). We have proposed recently that diminished binding affinity of BH_4 to the 'second' subunit of placental eNOS may play a role in the pathogenesis of pre-eclampsia through promoting the production of abnormally large quantities of O_2^- and peroxynitrite (Kukor *et al*., 2000).

Because BH_4 is sensitive to oxidative agents and can easily react with molecular oxygen even at ambient temperature, a potentially feasible approach to protect BH_4 under various conditions of oxidative stress could be the application of natural antioxidants such as ascorbic acid (vitamin C). According to recently reported experimental observations, ascorbic acid treatment of endothelial cells leads to an increase in intracellular BH_4 levels and this effect is due to chemical stabilization of the fully reduced form of the pterin (Heller *et al*., 1999, 2001; Huang *et al*., 2000; Baker *et al*., 2001). These findings validated the usefulness of vitamin C supplementation for preventing vascular damages and justified the administration of vitamin C in order to help prevent endothelial dysfunction or restore normal endothelial functions (Heitzer *et al*., 1996; Levine *et al*., 1996; Ting *et al*., 1996, 1997; Timimi *et al*., 1998; Gokce *et al*., 1999; Jeserich *et al*., 1999). In the same context, ascorbic acid may contribute to the maintenance of satisfactory eNOS activity in the human placenta, thereby reducing the risk of placental dysfunctions or improving placental functions in pathological pregnancies (Gulmezoglu *et al*., 1997; Chappell *et al*., 1999).

The present findings confirm the BH_4-stabilizing antioxidant effect of ascorbic acid and shed some light on its chemical mechanism. Although ascorbic acid itself is an antioxidant, it reacts readily with O_2 and produces H_2O_2, a potent oxidant. The BH_4-protective antioxidant effect of ascorbic acid may result from at least three mechanisms. (i) Elimination of O_2 from the solvent. In this respect, competition for O_2 as an antioxidant mechanism of ascorbic acid is unlikely, because simultaneous auto-oxidation of 25 µmol/l BH_4 and 100 µmol/l ascorbic acid could proceed in the same solution (Figure 5). Moreover, O_2 had a greater affinity toward BH_4 than toward ascorbic acid. (ii) Consumption of H_2O_2 via reduction. Indeed, ascorbic acid reacted with H_2O_2 readily (Figure 3A), and catalase dramatically decreased oxidation of ascorbic acid incubated with BH_4 (Figure 5), suggesting that H_2O_2 formed by the auto-oxidation of BH_4 is consumed by ascorbic acid. However, in the presence of O_2 dissolved in the incubation medium, BH_4 was relatively insensitive to oxidation by H_2O_2, as its auto-oxidation was only marginally increased by H_2O_2 (Figure 3B) and only slightly decreased by catalase (Figure 4B). Therefore, elimination of H_2O_2 by ascorbic acid does not contribute to the chemical stabilization of BH_4 to any significant extent. (iii) Direct reduction of BH_2 to regenerate BH_4 with the concomitant formation of dehydroascorbic acid. Our results suggest the existence of this mechanism. Thus, incubation of

1. $BH_4 + O_2 \rightarrow BH_2 + H_2O_2$

2. $HO–ASC–OH + O_2 \rightarrow O=ASC=O + H_2O_2$

3. $2\ HO–ASC–OH + 2\ H_2O_2 \rightarrow 2\ O=ASC=O + 4\ H_2O$

4. $HO–ASC–OH + BH_2 \rightarrow BH_4 + O=ASC=O$

Sum (1+4): $4\ HO–ASC–OH + 2\ O_2 = 4\ O=ASC=O + 4\ H_2O$

Figure 8. Proposed catalytic effect of tetrahydrobiopterin (BH_4) on ascorbic acid (ASC) oxidation. In the mixture of BH_4, ASC and O_2, an extensive oxidation of ascorbic acid takes place, whereas the concentration of BH_4 remains relatively unchanged.

ascorbic acid with BH_4 markedly increased (~3-fold) the rate of ascorbic acid oxidation and a major part of this increase was abolished by catalase, indicating that this part is mediated by H_2O_2 (Figure 5). Importantly, in the presence of catalase (which was shown to prevent completely the oxidation of ascorbic acid by H_2O_2), BH_4 still stimulated the rate of ascorbic acid oxidation by a factor of ~2 (Figure 5), demonstrating that a portion of the BH_4-stimulated ascorbic acid oxidation is not mediated by H_2O_2. Although BH_4 concentrations were not measured in these experiments, this part of increased ascorbic acid oxidation may be accounted for by the direct reduction of BH_2 (or rather the quininoid-BH_2 intermediate of the auto-oxidation process) to BH_4, since, under the conditions used, BH_2 was the only redox reaction partner for ascorbic acid. Because regenerated BH_4 is subject to repeated auto-oxidation, we propose that the reaction cycle recurs, continuously producing dehydroascorbic acid from ascorbic acid and O_2, while BH_4 concentrations remain approximately constant. Chemically, the overall process can be described as 'BH_4-catalysed oxidation of ascorbic acid' (Figure 8).

An important question is whether or not the in-vitro chemical stabilization of BH_4 by ascorbic acid is physiologically relevant. In our studies, 3 mmol/l ascorbic acid exerted an almost complete protective effect on 25 µmol/l BH_4 in the absence of EDTA, indicating that a 120-fold molar excess of ascorbic acid is sufficient for BH_4 protection. Average tissue concentrations of BH_4 in human placentae from first trimester and term pregnancies are 0.189 and 0.057 µmol/l respectively (Kukor *et al*., 2000). These values suggest that ascorbic acid concentrations as low as 7 µmol/l in term placentae or 23 µmol/l in primordial placentae can have significant protective effects *in vivo*. Importantly, these ascorbic acid concentrations are well within the range of vitamin C levels we measured in placental tissues, indicating that ascorbic acid may play a physiological role in the regulation of eNOS activity. In this respect, our in-vitro studies with placental eNOS enzyme clearly show that the protective effect of ascorbic acid on BH_4 can lead to elevated enzyme activity. In agreement with reports from other laboratories (Heller *et al*., 1999, 2001; Huang *et al*., 2000), the ascorbic acid dependent activity increase was not detectable at high, unphysiological BH_4 concentrations.

Vitamin C is evidently an important daily dietary supplement during pregnancy, and among other effects it may have a beneficial influence on placental and vascular functions. In some of the pre-eclamptic patients, failure to show significant improvements in response to regular vitamin C administration may stem from the malfunction of their placental eNOS enzyme. In a previous study (Kukor *et al*., 2000) we have found that the homogenates of seven out of 10 placentae obtained from pre-eclamptic pregnancies contain eNOS enzyme that is resistant to the stimulatory effect of 0.025–1.00 µmol/l BH_4, while the basal activity is sustained. This malfunction could not be corrected by elevated BH_4 concentrations, therefore in these cases increased vitamin C supply cannot alleviate the characteristic pre-eclamptic symptoms. On the other hand, in pre-eclamptic patients with functionally normal BH_4-responsive placental eNOS, one may expect beneficial protective and preventive effects from sustained vitamin C supplementation during pregnancy.

Acknowledgements

The help and interest of Miklós Sahin-Tóth and the technical assistance of Eszter Bérczi is greatly appreciated. This work was supported by Hungarian Research Fund (OTKA) grant T-29165.

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Submitted April 23, 2001; resubmitted August 29, 2001; accepted November 22, 2001