

ASCORBATE ENHANCES iNOS ACTIVITY BY INCREASING TETRAHYDROBIOPTERIN IN RAW 264.7 CELLS

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Abstract—Studies on the effect of ascorbic acid on inducible nitric oxide synthase (iNOS) activity are few and diverse, likely to be dependent on the species of cells. We investigated a role of ascorbic acid in iNOS induction and nitric oxide (NO) generation in mouse macrophage cell line RAW 264.7. Although interferon- γ (IFN- γ) alone produced NO end products, ascorbic acid enhanced NO production only when cells were synergistically stimulated with IFN- γ plus *Escherichia coli* lipopolysaccharide (LPS). Ascorbate neither enhanced nor decreased the expression of iNOS protein in RAW 264.7 cells, in contrast to the reports that ascorbic acid augments iNOS induction in a mouse macrophage-like cell line J774.1 and that ascorbate suppresses iNOS induction in rat skeletal muscle endothelial cells. Intracellular levels of tetrahydrobiopterin (BH₄), a cofactor for iNOS, were increased by ascorbate in RAW 264.7 cells. However, ascorbate did not increase GTP cyclohydrolase I mRNA, the main enzyme at the critical steps in the BH₄ synthetic pathway, expression levels and activity. Sepiapterin, which supplies BH₄ via salvage pathway, more efficiently enhanced NO production if ascorbate was added. These data suggest that enhanced activation of iNOS by ascorbic acid is mediated by increasing the stability of BH₄ in RAW 264.7 cells. © 2003 Elsevier Inc.

Keywords—Tetrahydrobiopterin, iNOS, Ascorbate, Sepiapterin, GTPCH, Free radicals

INTRODUCTION

Escherichia coli lipopolysaccharide (LPS) and cytokine administration to immortalized murine macrophage cell line RAW 264.7 [1–4] and rats [5–9] produced nitric oxide (NO) and its end product nitrite plus nitrate [10]. Nitrite plus nitrate formation from the activated macrophage cell line RAW 264.7 was enhanced over a very wide range of ascorbate concentrations (5–500 μ M) [11]. In mutant rats unable to synthesize ascorbic acid, ascorbic acid-supplied rats excreted more urinary nitrate after LPS stimulation [11,12]. These early studies suggest that ascorbic acid increases NO production from inducible nitric oxide synthase (iNOS) and probably contributes to preventing bacterial infection.

However, the mechanism for these effects is not clear. Ascorbic acid has been reported to augment iNOS induction after LPS plus IFN- γ stimulation in a mouse macrophage-like cell line J774.1 [13]. On the contrary,

ascorbic acid has been shown to suppress iNOS induction after LPS plus IFN- γ stimulation in rat skeletal muscle endothelial cells [14].

(6R)-5,6,7,8-Tetrahydrobiopterin (BH₄) is a critical cofactor required for activities of NOS isoforms [15–18]. Although de novo synthesis of BH₄ depends on expression levels of GTP cyclohydrolase I (GTPCH) [19], the turnover of BH₄ appears to be so rapid that the degradation of BH₄ [20,21] may attenuate iNOS activity in activated macrophages.

We aimed to reveal whether ascorbic acid increases iNOS induction or prevents degradation of BH₄ after LPS plus IFN- γ stimulation in macrophage cell line RAW 264.7.

MATERIALS AND METHODS

Cell culture

The mouse macrophage cell line RAW 264.7 (American Type Culture Collection, Rockville, MD, USA) was maintained in Dulbecco's modified Eagle medium (DMEM containing 5.5 mM glucose, Nissui Pharmaceu-

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tical Co., Ltd., Tokyo, Japan) supplemented with 10% heat-inactivated fetal calf serum (FCS), 4 mM L-glutamine, and 13 mM NaHCO₃. For experiments, cells (1×10^6 /ml) were resuspended with Eagle minimum essential medium (MEM, without phenol red, Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% heat-inactivated FCS, 1 mM pyruvate, 4 mM L-glutamine, 16 mM HEPES, and 13 mM NaHCO₃, and were activated with LPS alone (1 μ g/ml), IFN- γ alone (100 U/ml), or a combination of LPS plus IFN- γ .

Chemicals

Murine recombinant INF- γ was a generous gift from Shionogi (Osaka, Japan). L-ascorbic acid was obtained from Wako (Osaka, Japan), and LPS (type 0127:B8) and sepiapterin were purchased from Sigma Chemical Co.

NO determination

NO₂⁻ and NO₃⁻ in the culture medium were analyzed with an automated procedure based on the Griess reaction (Flow Injector Analyzer, TCI NOx, Tokyo Kasei Co., Tokyo, Japan) [3]. In brief, after reducing nitrate to nitrite through a copper-plated cadmium column, the absorbance at 540 nm was measured after the reaction with the Griess reagent. The value was expressed as the total amount of plasma NO end products, nitrite plus nitrate.

BH₄ determination

When sepiapterin (500 μ M) was added, quantification of BH₄ was unsuccessful by using high-performance liquid chromatography (HPLC) with fluorescence detection, because sepiapterin itself showed strong fluorescence. Therefore, BH₄ was measured using HPLC with electrochemical detection, as described previously [22,23]. We did not detect sepiapterin with electrochemical detection.

Briefly, cells from 100 mm dishes were collected with trypsin-EDTA, washed, lysed with pH 3 HPLC-grade water (0.5 ml) containing 100 μ M dithioerythritol (DTE) and 100 μ M diethylenetriaminepentaacetic acid (DTPA), and centrifuged at 11,000 rpm for 10 min. The supernatant was stored at -80°C until analysis. For analysis, the thawed samples were resolved with HPLC using a LC18 reverse-phase column (Shimadzu Corp., Kyoto, Japan) with mobile phase of 50 mM sodium acetate, 5.2 mM citrate, 60 μ M EDTA, 160 μ M DTE, and 5% methanol (pH 5.22) and detected using an EICOM ECD-100 electrochemical detector (Eicom, Kyoto, Japan) at an applied potential of +0.45 V. Under these conditions, biopterin

and dihydrobiopterin were not detected as described previously [23].

Western blot analysis

Protein expression was assayed by the Western blot analysis. Cells in a 60 cm dish were washed with ice-cold phosphate-buffered saline 24 h after stimulation. Cells were harvested by scraping and resuspended in lysis buffer containing 1% v/v NP40, 20 mM Tris (pH 7.7), 150 mM NaCl, 1 mM EDTA, and a mixture of protease inhibitors (Calbiochem, San Diego, CA, USA). After incubation at 4°C for 20 min, cells were centrifuged at 14,000 rpm for 10 min. The supernatant was analyzed for protein concentration, and an equal amount of cellular proteins (25 μ g/lane) was separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was then probed with monoclonal iNOS primary antibody (Transduction Laboratories, Lexington, KY, USA). Membrane-bound primary antibodies were visualized by using secondary antibodies conjugated with horseradish peroxidase and chemiluminescent substrate (Pierce, Rockford, IL, USA).

RT-PCR analysis of GTPCH mRNA expression

After the cells were treated for 24 h, RNA was extracted using the guanidine isothiocyanate method with TRIzol reagent, as described in the manufacturer's instructions (Life Technologies, Rockville, MD, USA). The RNA (1 μ g) was transcribed with oligo dT primer and Moloney murine leukemia virus reverse transcriptase (Life Technologies). The product of reverse transcription was subjected to PCR with Taq DNA polymerase (Life Technologies). The reaction mixture contained 20 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM deoxynucleotide mixture, 0.2 μ M of each PCR primer, and 25 U/ml Taq DNA polymerase. The primers used for human GTPCH mRNA were as follows: sense primer 5'-TACTCGTCCATCTGCTCTCG-3' and antisense primer 5'-GTCTTGCTGTTTCATTTTCTGC-3'. The PCR conditions were as follows: after initial melting at 94°C for 2 min, amplification was performed at 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s for 30 cycles, followed by a final extension at 72°C for 5 min. The PCR product was 452 base pairs. The PCR product was separated on 1.5% agarose gel and visualized with ethidium bromide under ultraviolet light. The gel image was captured with a charge-coupled device camera system (Bio-Craft Co., Ltd., Tokyo, Japan) and subjected to densitometry analysis using NIH image software (National Institutes of Health, Bethesda, MD, USA). The quantification of cDNA products was performed within the linear range of standard curve as shown in Figs. 5A and 5B.

Measurement of GTPCH activity

GTPCH activity was assayed as described [24]: 10^7 cells were collected and frozen at -80°C in 0.5 ml distilled water containing 5 mM dithioerythritol and 10 $\mu\text{g}/\text{ml}$ phenylmethylsulfonyl fluoride. Cells were broken by thawing, centrifuged at $13,000 \times g$ for 15 min at 4°C , and the supernatant (0.5 ml) was subjected to NAP-5 column (Pharmacia, Uppsala, Sweden) and eluted with 1 ml of 50 mM Tris-HCl, pH 7.8, containing 300 mM KCl, 2.5 mM EDTA, and 10% (v/v) glycerol. The eluate (250 μl) was mixed with 50 μl of 10 mM GTP, incubated for 60 min at 37°C in the dark, and was terminated by the addition of a mixture (40 μl) of 1 N HCl (20 μl) and 0.1 M I_2 in 0.25 mM KI (20 μl). After keeping the mixture at room temperature for 1 h and removing the precipitate by centrifugation at $13,000 \times g$ for 2 min at 25°C , excess iodine was reduced by the addition of 0.1 M fresh ascorbic acid (20 μl). The mixture was supplemented with 20 μl of 1 N NaOH and then incubated with 10 units of alkaline phosphatase at 37°C for 60 min in the dark. Neopterin in the supernatants was quantified with HPLC using a LC18 reverse-phase column (Shimadzu Corp.), which was eluted isocratically with a solvent of 20 mM NaH_2PO_4 (pH 3.0 with 85% H_3PO_4) in 5% (v/v) methanol at a flow rate of 0.8 ml/min and detected using a fluorescence detector (excitation at 350 nm, emission at 440 nm).

Statistical analysis

Data were given as mean \pm SE. A statistical analysis was performed using ANOVA followed by Fisher's PLSD (protected least significant difference) test, with a commercially available statistical package for the Macintosh personal computer (StatView-J, version 5, Abacus Concepts Inc., Berkeley, CA, USA). *P* values of less than .05 were considered statistically significant.

RESULTS

The mouse macrophage cell line RAW 264.7 was activated with LPS alone (1 $\mu\text{g}/\text{ml}$), IFN- γ alone (100 U/ml), or a combination of LPS plus IFN- γ (Fig. 1). NO synthesis was assayed by measuring the accumulation of the NO end products nitrite and nitrate in the medium. Although IFN- γ alone induced NO synthesis, NO production was synergistically enhanced when the cells were stimulated with the combination of IFN- γ and LPS. Next, we examined the effects of ascorbic acid on the NO synthesis. Ascorbic acid treatment (500 μM) distinctly enhanced NO production in the cells stimulated with the combination of LPS plus IFN- γ (Fig. 2A). We wondered which stimulus, either LPS or IFN- γ , is responsible for the enhancement in the NO production by

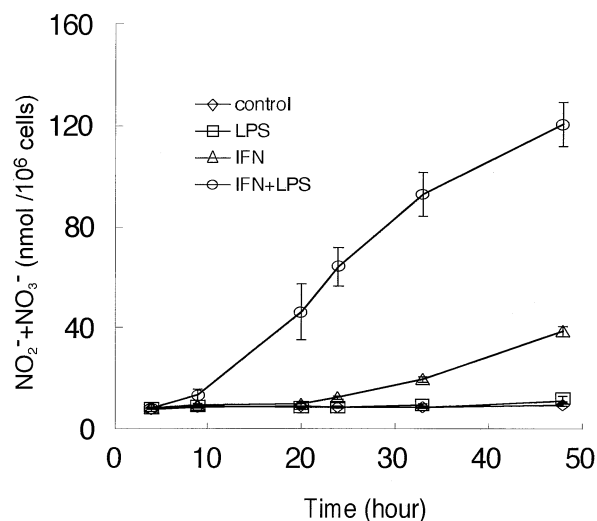


Fig. 1. Time course of NO end product nitrite plus nitrate in culture media of murine macrophage cell line RAW 264.7. After replacing with fresh supplemented MEM, cells ($1 \times 10^6/\text{ml}$) were stimulated with LPS alone (1 $\mu\text{g}/\text{ml}$), IFN- γ alone (100 U/ml), or a combination of LPS (1 $\mu\text{g}/\text{ml}$) plus IFN- γ (100 U/ml).

ascorbic acid. However, the effect was not observed in the cells stimulated with LPS or IFN- γ alone (Figs. 2B and 2C) and in nonstimulated cells (data not shown). From the results in Figs. 1 and 2, it can be assumed that the enhancement of NO synthesis by ascorbic acid does not depend on the nature of the stimuli but on the yield of NO, i.e., the activity of iNOS.

We then examined with Western immunoblot whether ascorbic acid (500 μM) augments iNOS protein expression. The expression of iNOS protein was not detected in nonstimulated cells. Although the stimulation with IFN- γ plus LPS induced the expression of iNOS protein, ascorbic acid (500 μM) did not enhance the expression of iNOS protein in the mouse macrophage cell line RAW 264.7 (Fig. 3). This is in contrast with the result in mouse macrophage cell line J774.1 where iNOS protein levels were increased approximately 2-fold [13].

Therefore, we examined whether ascorbic acid increases intracellular levels of BH_4 , the critical cofactor for NOS. BH_4 standard was detected electrochemically as a single peak and ascorbic acid did not interfere with quantification of the BH_4 peak (Fig. 4). Ascorbic acid (500 μM) increased the cellular BH_4 levels (Table 1).

To determine the mechanism for how ascorbic acid enhanced availability of BH_4 , we examined the mRNA expression and activity of GTPCH, the main enzyme in the critical steps in the BH_4 synthetic pathway. The mRNA expression of GTPCH was detected in a PCR cycle-dependent fashion (Figs. 5A and 5B), and ascorbic acid (500 μM) did not raise the GTPCH mRNA expression (Figs. 5A and 5C) and activity (Table 2) in nonstimulated cells. Even after stimulation with LPS plus

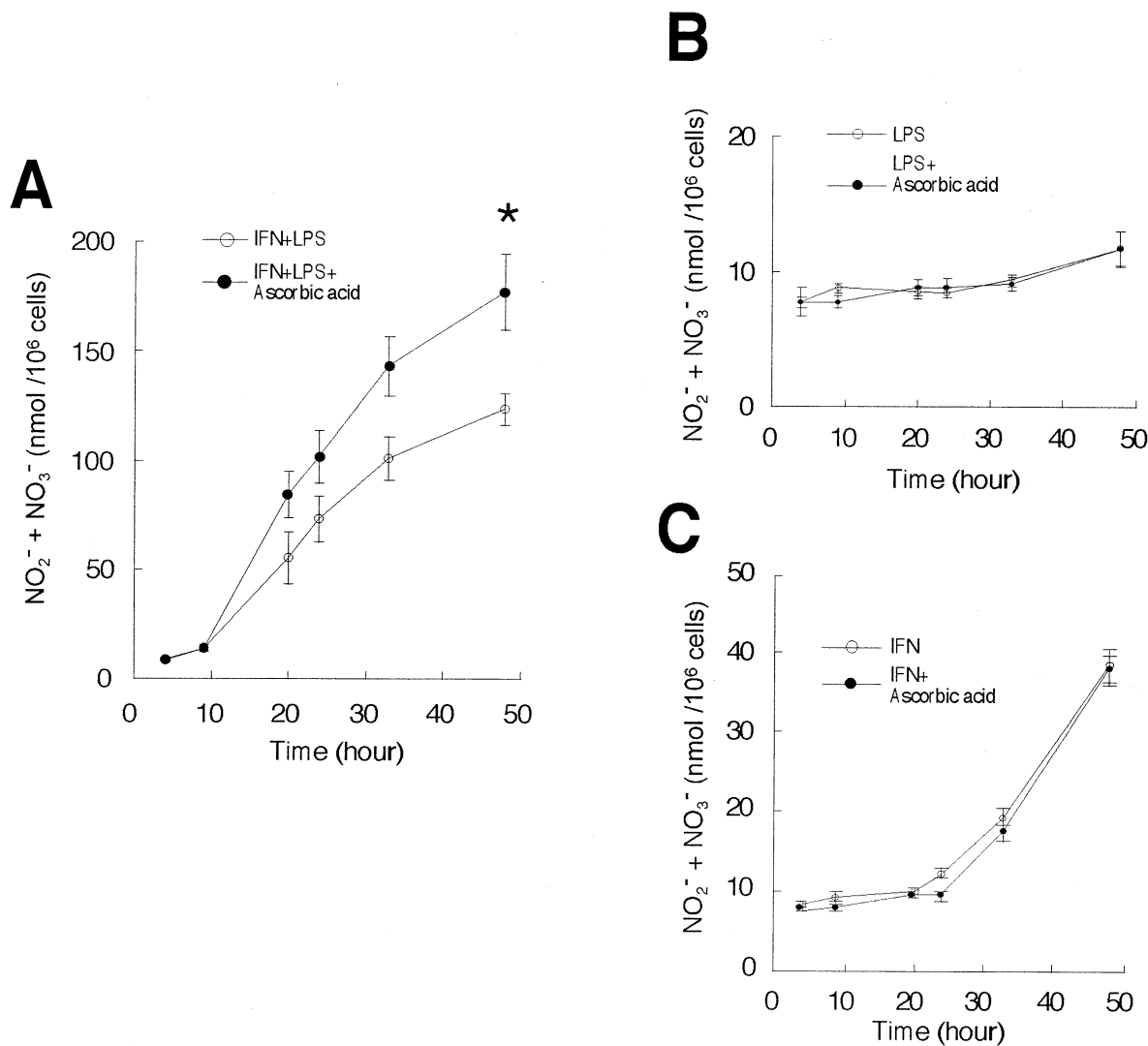


Fig. 2. The effects of ascorbic acid on NO synthesis in RAW 264.7 cells. After replacing with fresh supplemented MEM, ascorbic acid (500 μ M) was added to the incubation medium 2 h prior to the stimulation with LPS (1 μ g/ml) and/or IFN- γ (100 U/ml). The addition of ascorbic acid (500 μ M) significantly enhanced NO end products nitrite plus nitrate in the supernatant of cells (1×10^6 /ml) stimulated with (A) a combination of LPS plus IFN- γ . The effects of ascorbic acid (500 μ M) were not observed in the supernatant of cells (1×10^6 /ml) stimulated with (B) LPS or (C) IFN- γ alone. Values represent means \pm SE ($n = 4$). * $p < .05$ vs. cells without ascorbic acid.

IFN- γ , ascorbic acid did not raise the GTPCH mRNA expression (Fig. 5D) and activity (Table 2).

Sepiapterin has been reported to increase BH₄ through salvage pathway [17]. In fact, 500 μ M sepiapterin markedly increased intracellular BH₄ as compared with 500 μ M ascorbic acid in unstimulated cells after 24 h (Table 1). However, sepiapterin (500 μ M) enhanced the synthesis of NO end products in RAW 264.7 cells less efficiently than ascorbic acid (500 μ M) did 24 h after stimulation with LPS plus IFN- γ (Fig. 6). Conversely, sepiapterin (500 μ M) was more efficient for increasing NO end products in RAW 264.7 cells 24 h after stimulation with LPS plus IFN- γ , if ascorbic acid (500 μ M) was present. These results reveal that the augmentation

of iNOS activity in RAW 264.7 cells is mediated not only by supplying BH₄ through sepiapterin but also by increasing the stability of BH₄ with ascorbic acid. An in vitro experiment demonstrated that BH₄ degradation was markedly prevented by ascorbate (500 μ M) but not by NAC (500 μ M), as shown in Fig. 7.

DISCUSSION

In the present study, ascorbic acid enhanced NO production in murine macrophage cell line RAW 264.7 when cells produced large amount of NO by synergistic stimulation with IFN- γ plus LPS. Although ascorbic acid did not augment the induction of iNOS protein in the

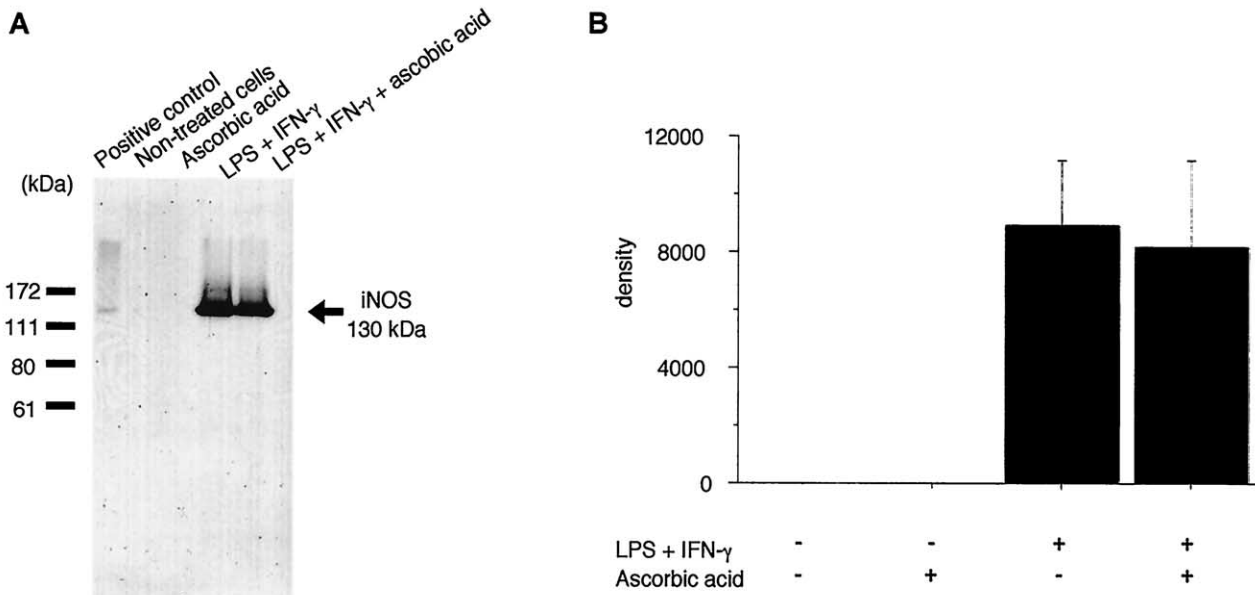


Fig. 3. Ascorbic acid does not affect iNOS protein expression in RAW 264.7 cells. (A) The results of immunoblot analyses using cell lysate (25 $\mu\text{g}/\text{lane}$) prepared from cells ($1 \times 10^6/\text{ml}$) that had been stimulated with a combination of LPS (1 $\mu\text{g}/\text{ml}$) plus IFN- γ (100 U/ml) for 24 h. Ascorbic acid (500 μM) was added 2 h prior to the stimulation. The arrow indicates immunoreactive bands corresponding to iNOS proteins (~ 130 kDa), at which we also detected signals using positive human macrophage-derived control samples provided by the supplier of the antibody. (B) Densitometric analysis of the immunoblots of iNOS protein ($n = 3$). A gel image was captured with a CCD camera system and subjected to densitometry analysis using NIH image software. There was no significant effect of ascorbic acid on the iNOS protein expression.

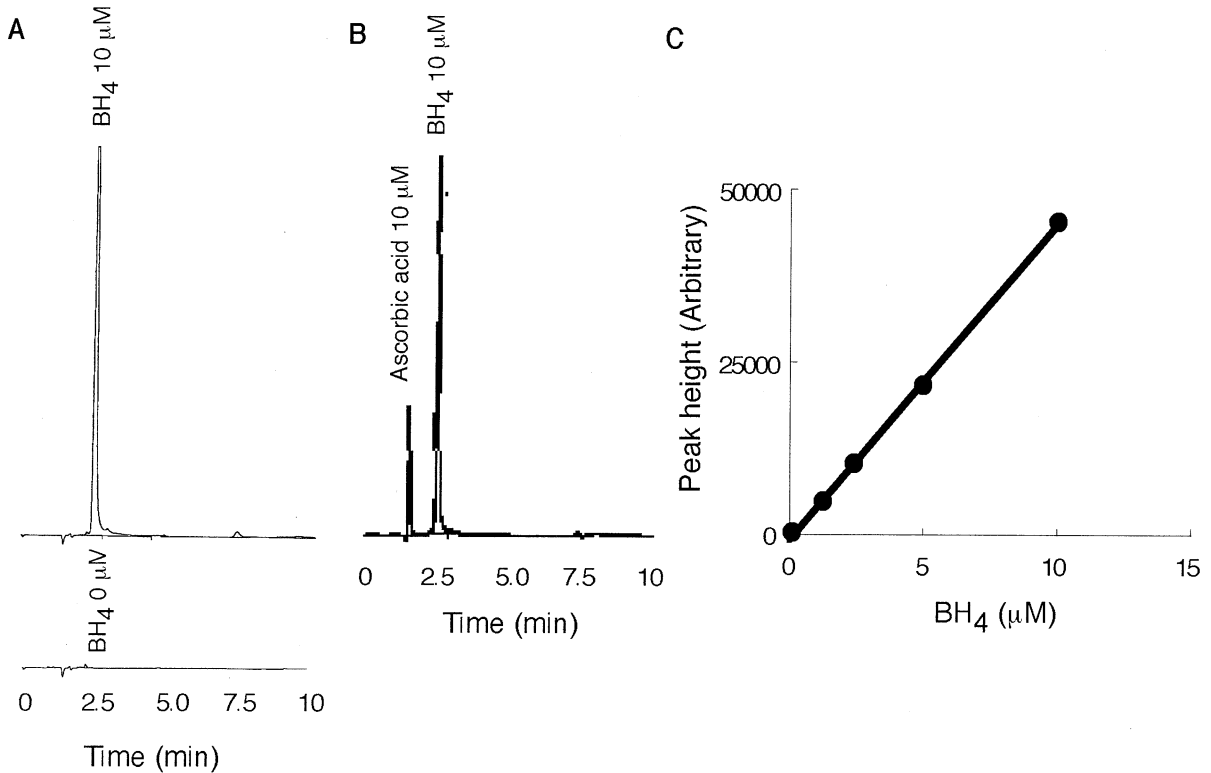


Fig. 4. BH₄ and ascorbic acid peaks and the standard curve of BH₄ with HPLC electrochemical detection. (A) The electrochemical chromatogram of standard 10 μM BH₄; (B) the electrochemical chromatogram of 10 μM ascorbic acid plus 10 μM BH₄; (C) the standard curve of BH₄.

Table 1. The Effects of Ascorbic Acid and/or Sepiapterin on Intracellular BH₄ in RAW 264.7 Cells

Treatment group	BH ₄ (pmol/10 ⁷ cells)
Control	422.1 ± 18.6
Ascorbic acid	573.8 ± 17.1*
Sepiapterin	10,451.4 ± 8.8* [†]
Sepiapterin + ascorbic acid	10,452.4 ± 5.4* [†]

RAW 264.7 cells (1 × 10⁶/ml) were treated with ascorbic acid (500 μM) and/or sepiapterin (500 μM) after replacing with fresh supplemented MEM. Intracellular BH₄ was determined after 24 h. Values represent mean ± SE (*n* = 3).

* *p* < .0001 vs. control.

[†] *p* < .0001 vs. ascorbic acid alone.

macrophages stimulated with IFN-γ plus LPS, ascorbic acid increased intracellular BH₄ levels in RAW 264.7 cells. However, ascorbic acid did not increase mRNA expression and activity of GTPCH, the rate-limiting enzyme in the synthesis of BH₄. Sepiapterin increased NO

end products in RAW 264.7 cells, however, less efficiently than ascorbic acid alone, although sepiapterin markedly increased BH₄ levels more than ascorbic acid. However, when ascorbic acid (500 μM) was supplied, sepiapterin (500 μM) was more effective at increasing NO end products more than sepiapterin or ascorbate alone in RAW 264.7 cells. These data suggest that the augmentation of NO production by ascorbic acid is mediated by preventing the oxidation of BH₄.

Ascorbic acid has been reported to increase iNOS mRNA and protein steady state levels approximately 2-fold in a mouse macrophage-like cell line J774.1 activated with LPS and IFN-γ [13,25]. On the contrary, although LPS (25 ng/ml) and IFN-γ (100 U/ml) induced iNOS protein expression and nitrite/nitrate formation in microvascular endothelial cell cultures derived from rat skeletal muscle, pretreatment of the endothelial cells with ascorbic acid decreased iNOS induction [14]. Why there is an essential difference in the mechanism for the

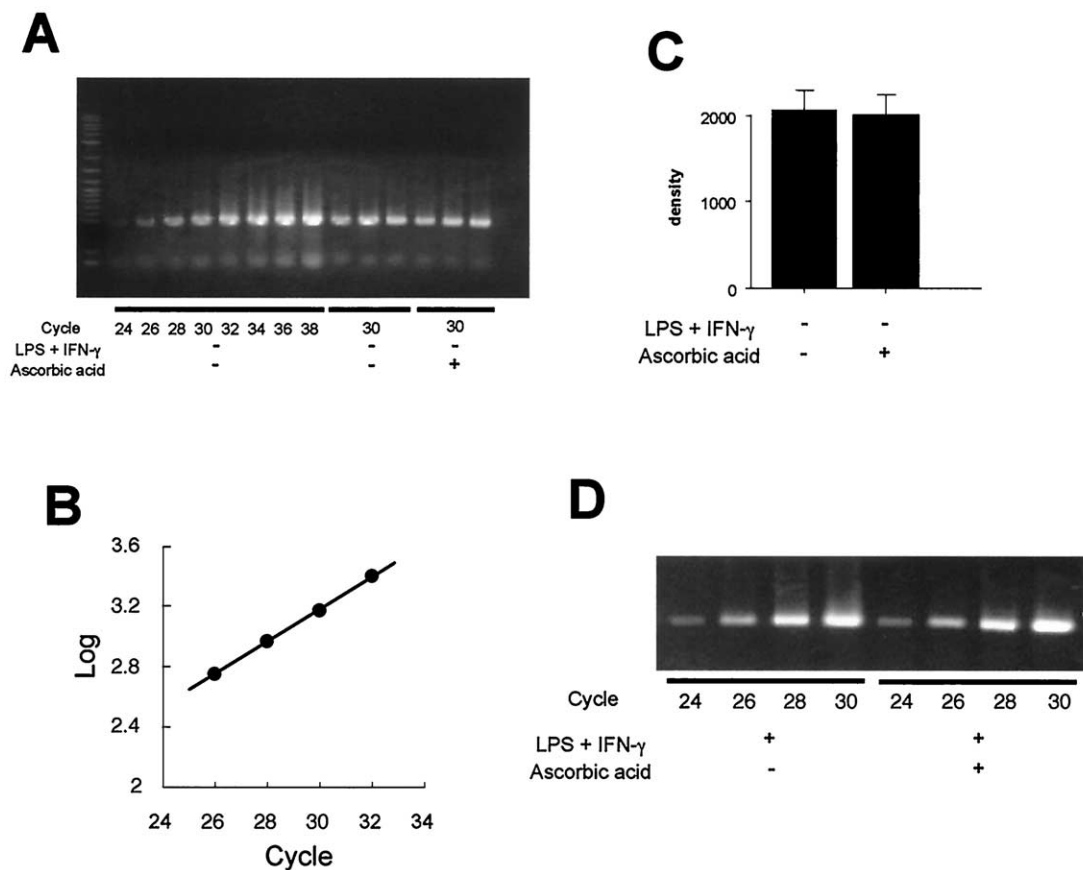


Fig. 5. The effects of ascorbic acid on the expression of GTPCH mRNA in RAW 264.7 cells. RNA was collected 24 h after the treatment of cells (1 × 10⁶/ml) with ascorbic acid (500 μM). (A) The representative gel image of semiquantitative RT-PCR analysis for GTPCH; (B) dependence of densitometric quantification of PCR products for GTPCH mRNA in control cells on PCR cycles; (C) the densitometric quantification of PCR products for GTPCH mRNA in cells without or with ascorbic acid (500 μM), before stimulation with LPS plus IFN-γ. Values represent mean ± SE (*n* = 3). No statistical significance was found. (D) PCR products for GTPCH mRNA in cells without or with ascorbic acid (500 μM) after stimulation with LPS (1 μg/ml) plus IFN-γ (100 U/ml) for 24 h.

Table 2. The Effects of Ascorbic Acid on GTPCH Activity in RAW 264.7 Cells Without or With Stimulation by LPS Plus IFN- γ

Ascorbic acid (μ M)	LPS plus IFN- γ	GTPCH (pmol/min/ 10^7 cells)
0	-	0.173 \pm 0.042
500	-	0.202 \pm 0.052
0	+	0.136 \pm 0.026
500	+	0.155 \pm 0.030

After replacing with fresh supplemented MEM, ascorbic acid was added to the incubation medium 2 h prior to the stimulation of RAW 264.7 cells (1×10^6 /ml) with LPS (1μ g/ml) plus IFN- γ (100 U/ml). GTPCH activity was determined 24 h after the stimulation. There was no significant effect of ascorbic acid on the GTPCH activity. Values represent means \pm SE ($n = 3$).

effect of ascorbic acid on iNOS induction is uncertain; it may be dependent on the species and kind of cells. However, our present findings revealed that, at least in murine macrophage cell line RAW 264.7, ascorbic acid did not affect iNOS protein, prevented BH₄ deficiency, and thus provided optimal conditions for cellular iNOS activity.

Endothelial NOS- (eNOS-) overexpressing (eNOS-Tg) mice were crossed with atherogenic apoE-deficient (apoE-KO) mice [26]. After 8 weeks on a high-cholesterol diet, the atherosclerotic lesion areas in the aortic sinus were unexpectedly increased by more than 2-fold in apoE-KO/eNOS-Tg mice compared with apoE-KO mice. eNOS dysfunction, demonstrated by lower NO

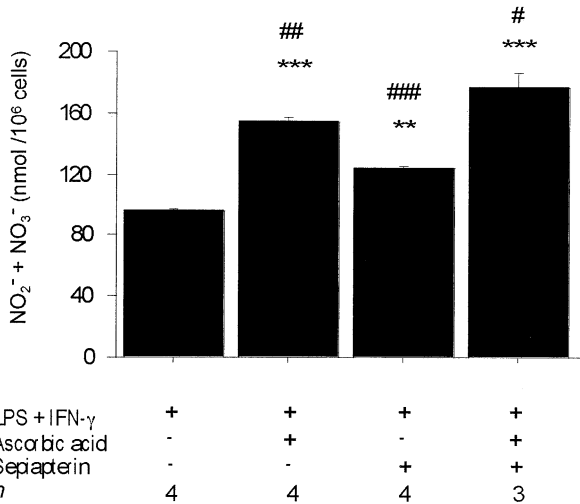


Fig. 6. The effects of sepiapterin and/or ascorbic acid on NO synthesis in RAW 264.7 cells. After replacing with fresh supplemented MEM, ascorbic acid (500 μ M) and/or sepiapterin (500 μ M) was added to the incubation medium 2 h prior to the stimulation of cells (1×10^6 /ml) with LPS (1μ g/ml) plus IFN- γ (100 U/ml) for 24 h. Values represent mean \pm SE. *** $p < .0001$; ** $p < .0005$ vs. cells without ascorbic acid or sepiapterin; ### $p < .0001$ vs. cells with ascorbic acid plus sepiapterin; ## $p < .0005$ vs. cells with sepiapterin; # $p < .005$ vs. cells with ascorbic acid.

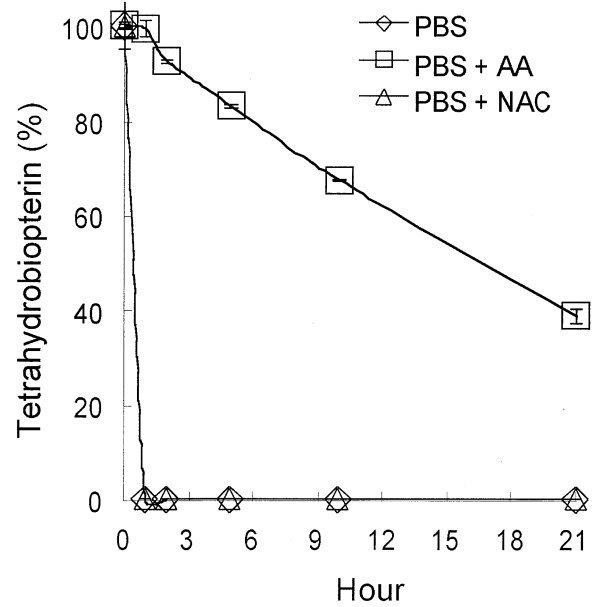


Fig. 7. The effects of ascorbic acid on in vitro BH₄ stability in aqueous solution. BH₄ (20 μ M) was added to phosphate-buffered saline (PBS, pH 7.4) and incubated at 37°C in the absence or presence of ascorbic acid. At the indicated times, 10 μ l aliquots were mixed with 90 μ l of pH 3 HPLC-grade water containing 100 μ M dithioerythritol (DTE) and 100 μ M DTPA; BH₄ was quantified as described in Materials and Methods. Data are expressed as percentages of values measured at zero time. AA = 500 μ M ascorbic acid; NAC = 500 μ M N-acetyl-cysteine. Values represent mean \pm SE ($n = 3$).

production relative to eNOS expression and enhanced superoxide production in the endothelium, was observed in apoE-KO/eNOS-Tg mice. Supplementation with BH₄ reduced the atherosclerotic lesion size in apoE-KO/eNOS-Tg mice. In contrast to the initial assumption that overexpression of eNOS can inhibit the development of atherosclerosis, the results indicate a BH₄ deficiency relative to overexpressed eNOS. A deficiency of BH₄ induces uncoupling of NOS that results in ROS generation [27–31]. Atherosclerotic changes also involve iNOS expression [32,33] and there have been a variety of reports regarding whether iNOS is harmful or beneficial [34–37]. As we have demonstrated in our present study, the induction of iNOS may accompany BH₄ deficiency under some in vivo conditions and may be harmful in such situations.

Ascorbic acid has been shown to stimulate endothelial NO synthesis in a time- and concentration-dependent fashion without affecting eNOS expression [23,38]. Pre-treatment of human umbilical vein endothelial cells with ascorbic acid for 24 h led to an up to 3-fold increase of intracellular BH₄ levels that was concentration-dependent and saturable at 100 μ M. Accordingly, the effect of ascorbic acid on Ca²⁺-dependent formation of citrulline and cGMP was abolished when intracellular BH₄ levels were increased by coincubation of endothelial cells with

sepiapterin, which is intracellularly converted into BH₄ via a salvage pathway. In our present study using murine macrophage cell line RAW 264.7, sepiapterin had an additive effect on iNOS activity compared with that of ascorbate. This may be because BH₄ supply for iNOS activity is not sufficient in the RAW 264.7 cells stimulated with LPS plus IFN- γ . Sepiapterin exactly increases the supply of BH₄ in unstimulated cells (Table 1). However, oxidative stress caused by stimulation with LPS plus IFN- γ [39] would have reduced the stability of BH₄. Ascorbate increases the stability of BH₄ (Fig. 7). Therefore, the combined effect of sepiapterin and ascorbate has increased iNOS activity more than sepiapterin alone.

In conclusion, ascorbic acid enhanced NO production in murine macrophage cell line RAW 264.7 stimulated with IFN- γ plus LPS. Ascorbic acid did not amplify the induction of iNOS protein in the stimulated cells and did not augment mRNA expression levels and activity of GTPCH. However, ascorbic acid increased intracellular BH₄ and an in vitro experiment demonstrated that BH₄ degradation was markedly prevented by ascorbate. Sepiapterin increased NO end products more efficiently if ascorbic acid was supplied. These results suggest that ascorbic acid improves insufficient BH₄ levels caused by inflammation and/or oxidative stress through increasing the stability of BH₄ and thereby augmenting iNOS activity.

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