FEBS Letters 520 (2002) 122–126 FEBS 26140

Ascorbate inhibits iNOS expression in endotoxin- and IFNγ-stimulated rat skeletal muscle endothelial cells

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Received 27 March 2002; revised 16 April 2002; accepted 22 April 2002

First published online 13 May 2002

Edited by Barry Halliwell

Abstract Lipopolysaccharide endotoxin and interferon-γ induced inducible nitric oxide synthase (iNOS) protein expression and nitrite/nitrate formation in microvascular endothelial cell cultures (ECs) derived from rat skeletal muscle. Pretreatment of ECs with ascorbate accumulated a large amount of ascorbate inside the cells and consequently decreased both intracellular oxidant level and iNOS induction. These effects of ascorbate were abolished in the presence of exogenous superoxide generated by xanthine oxidase/xanthine plus catalase but were not altered when *N*-nitro-L-arginine methyl ester was applied to inhibit nitric oxide synthesis. Ascorbate also attenuated the activation of transcription factor IRF-1 but not NFκB. These results indicate that ascorbate inhibits iNOS expression in ECs by an antioxidant mechanism independent of both NFκB activation and the reported negative feedback effect of nitric oxide. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Ascorbate; Inducible nitric oxide synthase; Endothelial cells; Oxidant; Sepsis

1. Introduction

As part of the systemic inflammatory response in sepsis, induction of inducible nitric oxide synthase (iNOS) by endotoxin (lipopolysaccharide, LPS) and inflammatory cytokines (e.g. interferon-γ, IFNγ) contributes to the bactericidal effects of phagocytes [1,2]. LPS and cytokines also induce iNOS in vascular endothelial cells (ECs) and smooth muscle cells; this induction is at least partially responsible for vascular hyporeactivity, hypotension and subsequent multiple organ failure [3,4]. Development of highly selective iNOS inhibitors for sepsis treatment has been fraught with difficulties and has not yielded the benefits expected [5,6]. Nevertheless, the process of iNOS induction remains a promising target for sepsis therapy.

Reactive oxygen species, superoxide in particular, have been implicated in the potentiation of iNOS induction in ECs and other cell types [7–9]. Depletion of the endogenous antioxidant ascorbate occurs during sepsis [10,11] and may sensitize cells to stimuli that induce iNOS. Recently, ascorbate administration was found to prevent the decrease of blood pressure and the maldistribution of capillary blood flow in skeletal muscle of septic rats [11]. These effects of ascorbate could have been mediated via inhibition of iNOS expression. The present study used microvascular ECs of rat skeletal muscle origin to evaluate the hypothesis that the antioxidant activity of ascorbate may prevent iNOS induction in ECs stimulated by LPS+IFNγ.

2. Methods

2.1. ECs from skeletal muscle

Microvascular ECs were isolated from the male Wistar rat extensor digitorum longus muscle using a cell-trapping technique as described by us [9]. ECs were cultured in medium M199 (Sigma, St. Louis, MO, USA; all chemical agents used in the current study were obtained from Sigma except those otherwise indicated) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Grand island, NY, USA), 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 30 μg/ml EC growth supplement (Collaborative Biomedical, Bedford, MA, USA) in a standard CO_2 incubator. EC identification was carried out as described by our laboratory [12]. Experiments were performed using confluent monolayers (passages three through eight) originating from at least three different rats for each experiment. ECs were treated with various agents (dissolved in the culture medium, i.e. the vehicle) as described in detail in figure legends.

2.2. Measurement of intracellular ascorbate

Ascorbate concentrations in ECs were assayed by acidic extraction and HPLC with electrochemical detection, according to a procedure described previously by us [13].

2.3. Measurement of nitrate and nitrite (NO_x)

NO_x content in the culture medium was measured to estimate the total nitric oxide (NO) production of cultured cells. An aliquot of the medium was filtered through a 10 000 Da molecular weight cutoff filter to eliminate proteins. Nitrate was converted to nitrite by nitrate reductase. Total nitrite was then measured using a total NO assay kit (Cayman, Ann Arbor, MI, USA). Briefly, the reduced sample was mixed with an equal volume of Griess reagent, and the absorbance at 545 nm was measured immediately. The NO_x concentration was determined by comparison to a standard curve based on serial dilution of a sodium nitrate standard. The standard curve was linear over a range of 3–50 μM ($r = 0.998$).

2.4. Western blot analysis

iNOS protein expression in ECs was determined by Western blot as previously described by us [9].

2.5. Measurement of intracellular oxidants

The intracellular oxidant levels in ECs were measured using dihydrorhodamine 123 (DHR123, Molecular Probes, Eugene, OR, USA),

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Abbreviations: NO, nitric oxide; iNOS, inducible nitric oxide synthase; ECs, endothelial cells; LPS, lipopolysaccharide; IFNγ, interferon-γ; L-NAME, *N*-nitro-L-arginine methyl ester; XO/X, xanthine oxidase/xanthine; SOD, superoxide dismutase

PII: S0014-5793(02)02804-1

an oxidant-sensitive fluorochrome, according to a procedure described in our previous study [9].

2.6. Nuclear extracts and electrophoretic mobility shift assay (EMSA)

Nuclear extracts of ECs were prepared as described previously [9]. For EMSA assay, double-stranded consensus oligonucleotides were end-labeled with [γ-^{32}P]ATP (Amersham Biosciences, Piscataway, NJ, USA) and T4 polynucleotide kinase (MBI Fermentas, Flamborough, ON, Canada). The sense sequences of oligoneleotides used were IRF-1 (5′-GGA AGC GAA AAT GAA ATT GAC T-3′) and NFκB (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The binding reaction was carried out as described previously [9]. IRF-1 and NFκB oligonucleotide specificity was verified by competition binding assay and supershift assay as described by us [9].

2.7. Data analysis

Data were expressed as mean ± S.E.M. values. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by a two-tailed Student *t*-test. Probabilities of $P < 0.05$ were considered significant.

3. Results

3.1. Ascorbate pretreatment accumulates ascorbate inside ECs

As shown in Fig. 1, non-preloaded ECs had undectable ascorbate while pretreatment of ECs with ascorbate for 24 h accumulated a substantial amount of ascorbate inside the cells. Assuming a cell water content of 4 μl/mg protein [12], the intracellular concentration of ascorbate was 5.3 ± 0.2 mM. The intracellular ascorbate was significantly decreased 6 h after the cells were transferred to ascorbate-free medium (bars 3 vs. 2) but was still markedly higher than in non-preloaded ECs (bars 3 vs. 1). Further, comparing the data at 6 h with that at 12 h after removal of extracellular ascorbate (bars 3 vs. 5) shows that intracellular ascorbate concentration decreased slowly between 6 and 12 h time points. LPS+IFNγ treatment did not change the intracellular concentration of ascorbate (bars 4 vs. 3 and bars 6 vs. 5), indicating that LPS+IFNγ did not cause ascorbate depletion.

3.2. Ascorbate pretreatment inhibits iNOS induction

Fig. 2 shows that non-stimulated ECs had undetectable iNOS protein with and without ascorbate pretreatment (lanes/bars 1 and 5). LPS alone had negligible effect on iNOS induction (lanes/bars 2 vs. 1), whereas IFNγ alone induced a substantial amount of iNOS protein (lanes/bars 3 vs. 1). Nevertheless the combination of LPS+IFNγ (i.e. the optimal concentration for iNOS induction in ECs [14]) caused even greater induction of iNOS (lanes/bars 4 vs. 1). However, the induction of iNOS by IFNγ alone or LPS+IFNγ was attenuated in ECs that had been loaded with ascorbate (lanes/bars 7 vs. 3 and 8 vs. 4). To confirm the results with ascorbate, we used the ascorbate derivative ascorbate-2-phosphate. Because the 2,3-enediol moiety of ascorbate is blocked with phosphate, ascorbate-2-phosphate is resistant to oxidation and generates less oxygen radicals in the medium than does extracellular ascorbate [15]. As shown in Fig. 3, pretreatment of ECs with ascorbate-2-phosphate decreased the iNOS protein induced by LPS+IFNγ (lanes/bars 4 vs. 2) to the same extent as did ascorbate (lanes/bars 4 vs. 3). Co-administration of superoxide dismutase (SOD), the superoxide anion scavenger, diminished LPS+IFNγ-induced iNOS expression (lanes/bars 5 vs. 2) to the same extent as did ascorbate preloading (lanes/bars 5 vs. 3). Ascorbate alone

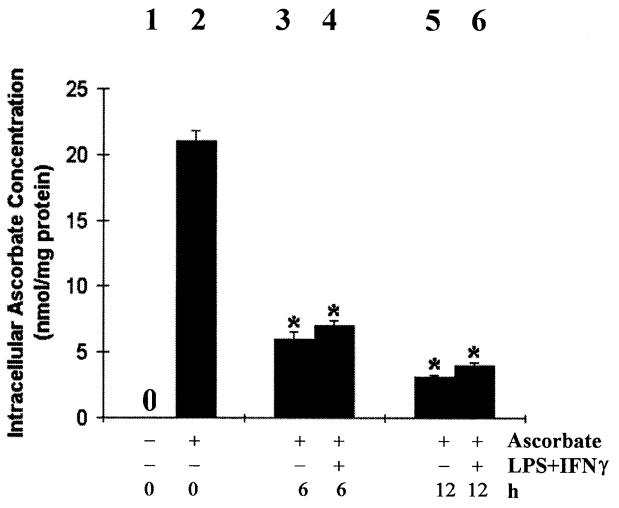

Fig. 1. Intracellular ascorbate concentration in ECs after ascorbate pretreatment. ECs were incubated with vehicle or 100 μM ascorbate for 24 h, then treated in ascorbate-free medium with LPS (25 ng/ml)+IFNγ (100 U/ml) or without LPS+IFNγ for the indicated times. Ascorbate concentrations in the cells were assayed by acidic extraction and HPLC with electrochemical detection. Shown are means ± S.E.M. for three separate experiments with duplicate determinations in each. * indicates $P < 0.05$ compared to both the vehicle-treated and ascorbate-treated groups at 0 h.

did not induce iNOS protein expression and did not affect cell viability as assessed by Trypan blue exclusion assay (data not shown).

3.3. Ascorbate acts through decreasing intracellular oxidant stress

Fig. 4 shows that ascorbate pretreatment significantly decreased the levels of intracellular oxidants in both non-stimulated and LPS+IFNγ-stimulated cells (bars 2 vs. 1 and 4 vs. 3 in Fig. 4B). The decrease observed in the LPS+IFNγ-stimulated ECs was associated with marked inhibition of iNOS expression (lanes 4 vs. 3 in Fig. 4A). To identify the oxidants that ascorbate targets, LPS+IFNγ-treated ECs were exposed to exogenous superoxide generated by xanthine oxidase/xanthine (XO/X) plus catalase. Since the XO/X system also makes hydrogen peroxide and hydroxyl radical [16], we added catalase to reduce hydrogen peroxide and to block hydroxyl radical generation. Superoxide caused a significant increase of intracellular oxidant stress (bars 5 vs. 3 in Fig. 4B) that was paralleled by an enhanced iNOS expression (lanes 5 vs. 3 in Fig. 4A). Moreover, superoxide abolished the effect of preloaded ascorbate on iNOS induction (lanes 6 vs. 4 in Fig. 4A) by increasing the intracellular oxidant stress (bars 6 vs. 4 in Fig. 4B). Superoxide alone did not induce iNOS expression in ECs that had not been stimulated by LPS+IFNγ (data not shown). These results indicate that ascorbate prevents iNOS induction by scavenging superoxide, since its effect was abrogated by excess superoxide.

3.4. Ascorbate effect is not mediated by NO

'iNOS induction in ECs can be inhibited by NO donor [9]. Therefore, we investigated whether scavenging of superoxide by ascorbate increases endogenous NO to levels that suppress iNOS induction. As shown in Fig. 5, ascorbate pre-

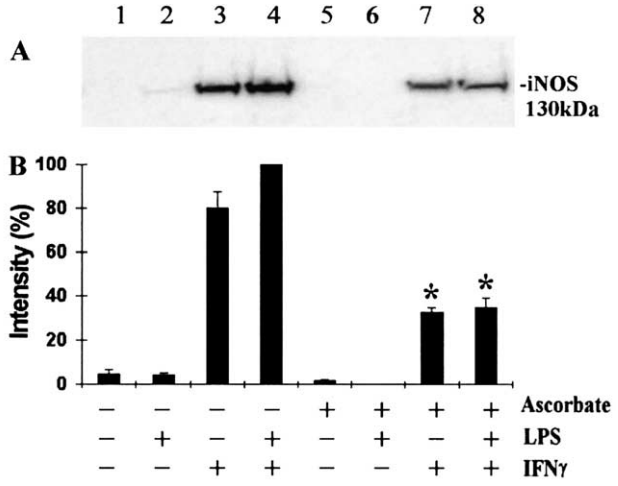

Fig. 2. Ascorbate pretreatment inhibits iNOS induction by IFNγ and LPS+IFNγ. ECs were incubated with vehicle or 100 μM ascorbate for 24 h and subsequently were incubated with vehicle, LPS (25 ng/ml), IFNγ (100 U/ml), or LPS+IFNγ for 12 h. Shown are an iNOS Western blot example (panel A) and the summary of protein band intensities (panel B; expressed as percentages of values for the LPS+IFNγ-stimulated group; mean ± S.E.M. values for three experiments). * indicates $P < 0.05$ for the effect of ascorbate compared to the relevant ascorbate-free group.

treatment decreased iNOS expression and NO_x formation in ECs stimulated by LPS+IFNγ (lanes/bars 4 vs. 3). The NOS inhibitor *N*-nitro-L-arginine methyl ester (L-NAME) prevented NO_x formation (bars 5 vs. 3) but enhanced iNOS expression (lanes 5 vs. 3). However, the inhibition of iNOS expression by ascorbate occurred even in the presence of L-NAME (i.e. in the absence of endogenous NO) (lanes/bars 6 vs. 5) as well as in the presence of oxyhemoglobin (10 μM) that scavenges NO (data not shown). Therefore, our results are not consistent with the hypothesis that inhibition of iNOS expression by ascorbate is mediated by negative feedback of NO.

3.5. Ascorbate modulation of IRF-1 and NFκB

Since regulation of iNOS expression occurs mainly at the transcriptional level [7,8] and IRF-1 and NFκB are important in iNOS gene activation [17,18], the current study examined the effect of ascorbate on activation of both these transcription factors. IFNγ or LPS+IFNγ, but not LPS alone, caused a significant increase in activation of IRF-1 (lanes/bars 3,4 vs. 1 in Fig. 6). This activation was associated with increased iNOS expression (lanes/bars 3,4 vs. 1 in Fig. 2). A comparison of lanes/bars 7,8 against lanes/bars 3,4, respectively, in Fig. 6, reveals much less IRF-1 activation in ascorbate-loaded cells, suggesting that ascorbate reduces iNOS induction (lanes/bars 7,8 in Fig. 2) at the IRF-1 level.

NFκB was activated after LPS+IFNγ exposure (lanes/bars 1 vs. 4 in Fig. 7). This activation was consistent with iNOS protein expression (lanes/bars 4 vs. 1 in Fig. 2). However, NFκB was also activated after LPS alone (lanes/bars 2 vs. 1 in Fig. 7) without concomitant iNOS expression (lanes/bars 2 vs. 1 in Fig. 2). Thus, NFκB activation was not sufficient for iNOS induction. Ascorbate had no effect on NFκB activation (lanes/bars 6,8 vs. 2,4 in Fig. 7). Therefore, the modulating effect of ascorbate on iNOS induction (Fig. 2) was unlikely to be mediated at the NFκB level.

4. Discussion

Since oxidation of ascorbate in culture medium produces hydrogen peroxide that may affect cellular function [19], we pretreated the cells with ascorbate or ascorbate-2-phosphate prior to LPS+INFγ exposure. Pretreatment with either form of ascorbate inhibited the induction of iNOS protein and NO_x production by LPS+IFNγ. This effect was due to an increase in the intracellular ascorbate concentration. ECs did not contain ascorbate when grown under normal culture conditions (Fig. 1, bar 1) but accumulated it to millimolar levels when incubated with a physiologically relevant ascorbate concentration (Fig. 1, bar 2). Although rat hepatocytes can synthesize ascorbate [20], ECs lack this ability. The absence of intracellular ascorbate may account for the oxidative stress observed in ECs without ascorbate pretreatment (Fig. 4, bars 1 and 3). Pretreatment with ascorbate increased the intracellular concentration of ascorbate (Fig. 1, bar 2) and consequently decreased the intracellular oxidant stress in both non-stimulated and LPS+IFNγ-stimulated ECs (Fig. 4B). The decrease observed in the LPS+IFNγ-stimulated ECs was associated with marked inhibition of iNOS expression. These antioxidant effects of ascorbate could be abrogated by excessive superoxide, indicating that ascorbate prevents iNOS induction by scavenging the intracellular superoxide. Blockade of endogenous NO synthesis by L-NAME did not alter the effect of ascorbate, suggesting that ascorbate does not inhibit iNOS induction by altering intracellular NO concentration. Taken together, these observations indicate that it is the antioxidant action of intracellular ascorbate that prevents superoxide-potentiated iNOS induction.

The promoter region of rat iNOS gene contains binding sites for a number of transcription factors, including NFκB and IRF-1 [17,18]. We found no effect of ascorbate on LPS- or LPS+IFNγ-induced activation of NFκB. Similarly, ascorbate did not alter NFκB activation by TNFα in human aortic ECs [21]. Other antioxidants also failed to inhibit NFκB acti-

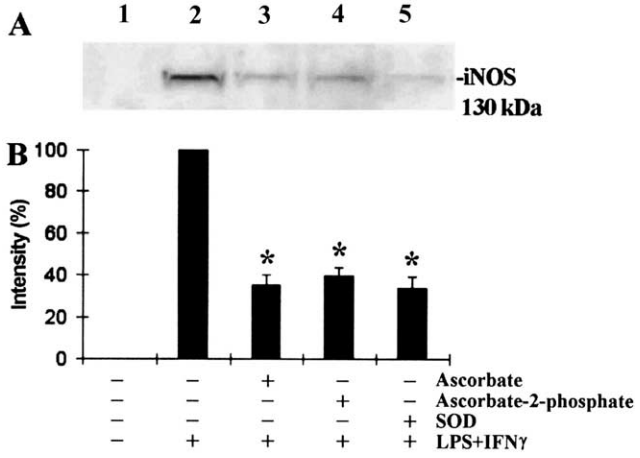

Fig. 3. Ascorbate-2-phosphate and SOD inhibit iNOS induction by LPS+IFNγ. ECs were incubated with vehicle, 100 μM ascorbate, or 100 μM ascorbate-2-phosphate for 24 h. The cells were then incubated with vehicle, LPS+IFNγ, or LPS+IFNγ plus SOD (500 U/ml) for 12 h. Shown are an iNOS Western blot example (panel A) and the summary of protein band intensities (panel B; expressed as percentages of values for the LPS+IFNγ-stimulated group; mean ± S.E.M. values for three experiments). * indicates $P < 0.05$ compared to the LPS+IFNγ group.

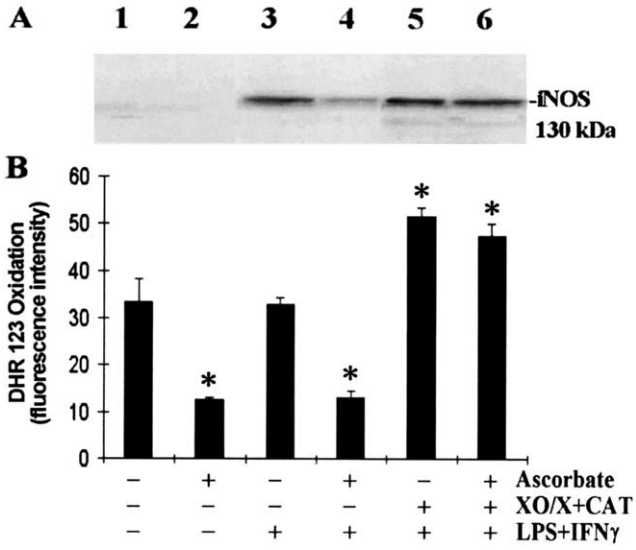

Fig. 4. Superoxide abolishes the effect of ascorbate pretreatment on iNOS induction by LPS+IFNγ. ECs were incubated for 24 h with vehicle or 100 μM ascorbate. Next they were incubated with vehicle, LPS+IFNγ, or the combination of LPS+IFNγ, XO/X (10 mU per ml/100 μM) and catalase (CAT; 5000 U/ml), either for 12 h to induce iNOS protein or for 6 h in the presence of 20 μM DHR123. The oxidized DHR123 was measured by fluorescence spectrophotometry. Shown are an iNOS Western blot example of three experiments (panel A) and the summary of DHR123 fluorescence data (panel B; mean ± S.E.M. values for four experiments with triplicate determinations in each). * indicates $P < 0.05$ compared to the control group that did not receive ascorbate, superoxide or LPS+IFNγ.

vation in macrophage cells stimulated by LPS or LPS+IFNγ, but nevertheless prevented iNOS induction [22,23]. However, the present experiments revealed that ascorbate inhibition of iNOS induction was associated with attenuation of IRF-1 activation. This effect of ascorbate in ECs is consistent with the observation in a macrophage cell line that antioxidants (5,7-dihydroxyflavone; 3,4-dichloroisocoumarin; *N*-acetyl-5-

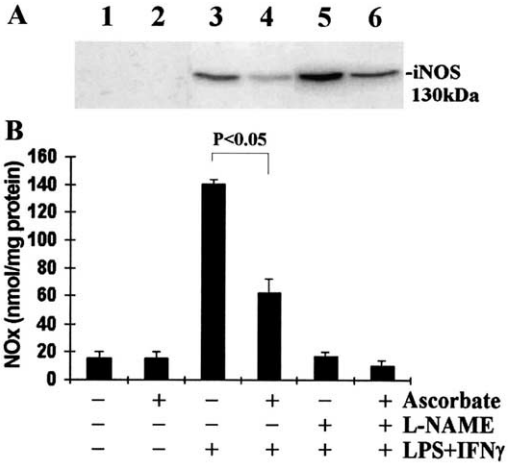

Fig. 5. Ascorbate inhibition of iNOS induction is not mediated by NO. ECs were incubated for 24 h with vehicle or 100 μM ascorbate. Next, they were incubated for 12 h with vehicle, LPS+IFNγ, or the combination of LPS+IFNγ and L-NAME (5 mM). NO_x in the culture medium was measured by Griess reaction. Shown are an iNOS Western blot example of three experiments (panel A) and the summary of NO_x levels (panel B; mean ± S.E.M. values for 4 experiments with duplicate determinations in each).

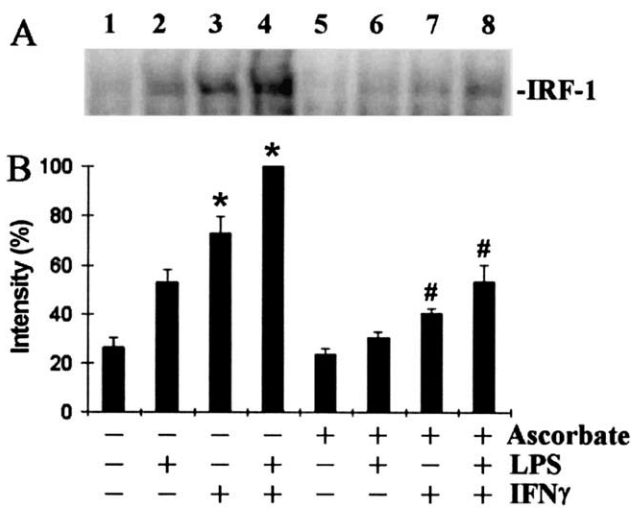

Fig. 6. Effect of ascorbate on LPS, IFNγ, or LPS+IFNγ-stimulated activation of IRF-1. ECs were incubated for 24 h with vehicle or 100 μM ascorbate. Subsequently they were incubated with vehicle, LPS, IFNγ, or LPS+IFNγ for 12 h. Nuclear proteins were extracted and reacted with specific oligonucleotides. Shown are an IRF-1 EMSA example (panel A) and the summary of IRF-1-specific band intensities (panel B; expressed as percentages of values for the LPS+IFNγ-stimulated group; mean ± S.E.M. values for three experiments). * indicates $P < 0.05$ for the effect of IFNγ or LPS+IFNγ compared to the non-treated group (bar 1); # indicates $P < 0.05$ for the effect of ascorbate compared to the relevant ascorbate-free group.

hydroxytryptamine) blunt the DNA-binding activity of IRF-1 that mediates iNOS induction by LPS+IFNγ [24].

While inhibiting iNOS expression, ascorbate can preserve the activity of constitutive NOS [22,25]. This mechanism may be important in sepsis in maintaining the physiological functions of NO, such as preventing leukocytes' adherence to

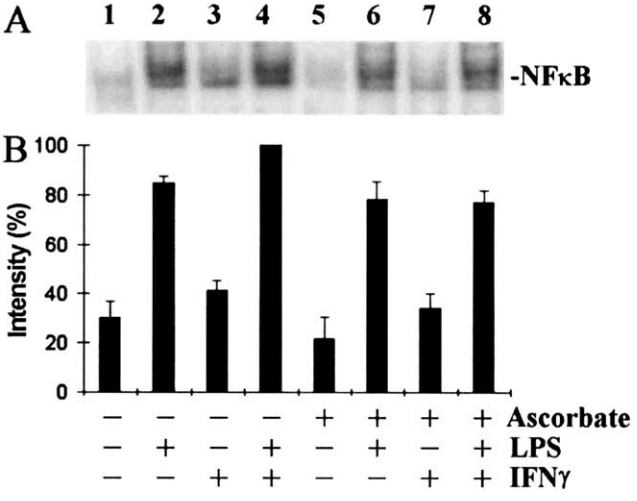

Fig. 7. Effect of ascorbate on LPS, IFNγ, or LPS+IFNγ-stimulated activation of NFκB. ECs were incubated for 24 h with vehicle or 100 μM ascorbate. Subsequently they were treated with vehicle, LPS, IFNγ, or LPS+IFNγ for 12 h. Nuclear proteins were extracted and reacted with specific oligonucleotides. Shown are a NFκB EMSA example (panel A) and the summary of NFκB-specific band intensities (panel B; expressed as percentages of values for the LPS+IFNγ-stimulated group; mean ± S.E.M. values for 3 experiments). Ascorbate had no effect on NFκB levels in any of the treatment groups.

endothelium [26]. In addition, ascorbate may prevent direct injury by oxidants that have been implicated in the pathogenesis of sepsis [27,28]. Thus, ascorbate treatment may be a new way to selectively inhibit iNOS induction for sepsis therapy. We conclude that pretreatment of microvascular ECs with ascorbate blunts iNOS induction by LPS+IFNγ by reducing intracellular oxidant stress.

Acknowledgements: This work was supported by the Canadian Institutes of Health Research, the Heart and Stroke Foundation of Ontario, and the Lawson Health Research Institute.

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