Expression of Inducible Nitric Oxide Synthase in Bovine Corneal Endothelial Cells and Keratocytes In Vitro After Lipopolysaccharide and Cytokines Stimulation

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Purpose. To determine whether bovine corneal endothelial (BCE) cells and keratocytes express the inducible form of nitric oxide synthase (NOS) after exposure to cytokines and lipopolysaccharide (LPS), and to study the regulation of NOS by growth factors.

Methods. Cultures of bovine corneal endothelial cells and keratocytes were exposed to increasing concentrations of LPS, interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α). At selected intervals after exposure, nitrite levels in the supernatants were evaluated by the Griess reaction. Total RNA was extracted from the cell cultures, and messenger RNA levels for inducible NOS (NOS-2) were measured by reverse transcription–polymerase chain reaction (RT–PCR).

Results. Exposure of BCE cells and keratocytes to LPS and IFN-γ resulted in an increase of nitrite levels that was potentiate by the addition of TNF-α. Analysis by RT–PCR demonstrated that nitrite release was correlated to the expression of NOS-2 messenger RNA in BCE cells and keratocytes. Stereoselective inhibitors of NOS and cycloheximide inhibited LPS–IFN-γ-induced nitrite release in both cells, whereas transforming growth factor-β (TGF-β) slightly potentiated it. Fibroblast growth factor-2 (FGF-2) inhibited LPS–IFN-γ-induced nitrite release and NOS-2 messenger RNA accumulation in keratocytes but not in BCE cells.

Conclusions. The results demonstrate that in vitro activation of keratocytes and BCE cells by LPS and cytokines induces NOS-2 expression and release of large amounts of NO. The high amounts of NO could be involved in inflammatory corneal diseases in vivo. Invest Ophthalmol Vis Sci. 1997;38:2045–2052.

Nitric oxide (NO) is a free radical, synthesized from L-arginine by nitric oxide synthase (NOS). Nitric oxide is involved in such diverse processes as neurotransmission, vasodilation, host defense, and inflammation.[1–4] The existence of at least three different forms of NOS, each coded by a specific gene, has been demonstrated. Two forms of NOS are calcium–calmodulin-dependent and are constitutively expressed in endothelial cells and in the brain.[1,2] In addition, a wide array of cell types can express the inducible form of NOS (NOS-2 or iNOS) when stimulated by endotoxins or cytokines.[3,4] This expression was demonstrated in rodent macrophages[5] and in various other cells,[4] including some ocular cells.[6–11] Cultured retinal pigmented epithelial (RPE) cells stimulated by lipopolysaccharide (LPS) and interferon-γ (IFN-γ) express the messenger RNA (mRNA) for NOS-2 and released nitrite, the stable end-product of NO, into the culture supernatant.[6–9] In the retina, the Müller glial cells,[10] the capillary endothelial cells, and the pericytes,[11] also express NOS-2 after treatment with LPS and cytokines. In the anterior segment of the eye, we recently reported that epithelial cells from the iris-ciliary body could express NOS-2 mRNA in the rat during endotoxin-induced uveitis.[12,13] However, to our knowledge, corneal cells have never been reported to express NOS-2 and to release high amounts of NO in vitro or in vivo.

The cornea, an avascular tissue, consists of several layers of cells.[14,15] The principal cell types constituting the cornea are epithelial cells, stromal cells termed

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Supported in part by grants from INSERM, Paris, France.
Proprietary interest category: N.
Submitted for publication January 7, 1997; revised May 8, 1997; accepted May 12, 1997.
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keratocytes, and endothelial cells. Keratocytes in the cornea are responsible for the synthesis of collagen fibrils and intercellular matrix and for stromal repair after injury or infection. The corneal endothelium contributes to the corneal transparency by forming a barrier between the aqueous humor and the corneal stroma and by its function as an active metabolic pump.[14,15]

In this article, we have investigated the effect of endotoxin and cytokines on NOS induction in the cornea by evaluating nitrite release and NOS-2 mRNA accumulation in subcultured bovine corneal endothelial BCE cells and keratocytes.

MATERIALS AND METHODS

Cell Cultures

Bovine eyes from a local slaughterhouse were processed at the laboratory within 3 hours of enucleation. The corneas were dissected, and the endothelium was scraped off with a dull-edged spatula. The endothelium fragments obtained were placed in Petri dishes containing Dulbecco's modified essential medium (DMEM), supplemented with 10% fetal calf serum (FCS; GibcoBRL, Gergy–Pontoise, France), fungizone (2.5 μg/ml), gentamycin (50 μg/mg), and L-glutamine (2 mM). Cultures were typically confluent in 15 days.[16] Cells were used between the second and the fourth passages.

For the culture of corneal stromal cells (keratocytes), the epithelial layer was scraped away and the stroma was cut in small fragments. These fragments were incubated for 30 minutes in collagenase (200 U/ml at 37°C) centrifuged, and digested for 15 minutes at 37°C with trypsin 0.05%–EDTA 0.02%. After centrifugation, cells were suspended in DMEM as before and seeded in 25-cm^2 culture flasks.[17] Cultures, in a humidified atmosphere of 5% CO_2–95% air at 37°C, were confluent after 8 to 10 days. Cells were used between the second and the fifth passages.

Chemicals and Cytokines

Lipopolysaccharide from *Salmonella typhimurium* (L 6143) was purchased from Sigma (St. Quentin Fallavier, France); human recombinant interleukin (IL)-1$_\beta$ and human recombinant tumor necrosis factor-α (TNF-α) from Pepro Tech (TEBU, Le Perray en Yvelines, France); and human transforming growth factor-β_2 (TGF-β_2) from R & D Systems (Abingdon, UK). Bovine recombinant IFN-γ was generously provided by Dr T. Ramp (Ciba–Geigy, Basel, Switzerland), and human recombinant fibroblast growth factor-2 (FGF-2) was a generous gift from Carlo Erba (Italy).

Formation of Nitrite

The BCE cells and keratocytes were seeded at a density of 5.10^4 per well in 12-well culture plates (Falcon Laboratories, PolyLabo, Paris, France). At confluency, cells were treated with LPS, other cytokines, or both, in fresh culture medium. After 12, 24, 48, 72, or 96 hours of incubation, the nitrite concentration was determined in cell-free culture supernatants, using the spectrophotometric method based on the Griess reaction.[18] Briefly, samples were reacted with 1% sulfanilamide and 0.1% naphthylethylenediamine at room temperature for 10 minutes, and the nitrite concentration was determined by absorbance at 540 nm in comparison with that of standard solutions of sodium nitrite prepared in DMEM.

RNA Isolation and Reverse Transcription–Polymerase Chain Reaction Analysis

Total RNA was extracted from cultured cells treated by cell lysis in guanidine isothiocyanate, followed by phenol acid extraction.[19] One microgram of RNA underwent reverse transcription for 90 minutes at 42°C with 200 U of Superscript Moloney murine leukemia virus reverse transcription (Life Technologies SARL, Eragny, France), using random hexamers, and 2 μl of cDNA was added to each RT–PCR reaction, as previously described.[12] Amplification was performed as follows: 94°C for 2 minutes; 26 cycles for reduced glyceraldehyde–phosphate dehydrogenase (GAPDH) and 30 cycles for NOS-2 (number of cycles that were below the saturating conditions) of 94°C for 30 seconds; 55°C for 30 seconds; 72°C for 45 seconds; and 72°C for 2 minutes. To verify that equal amounts of RNA were added in each RT–PCR within an experiment and to verify a uniform amplification process, GAPDH mRNA was also reverse transcribed and amplified for each sample. The amplified fragments were separated on 1.5% agarose gel, and bands were visualized with ethidium bromide. The intensity of the bands was quantified using densitometric measurements. A high-resolution camera coupled to an image processor (Ultra-Lum Inc., Carson, CA) and driven by One Descan densitometric software (Scanalytics, Billerica, MA) was used to perform densitometric measurements. Restriction analysis was used to confirm the identity of NOS-2 RT–PCR products, which have unique restriction sites for *BamH1*. Aliquots of RT–PCR products were digested for 1 hour at 37°C, restriction digests were run on agarose gel, and bands were visualized with ethidium bromide to verify that the actual size of the bands corresponded to the predicted size.

The nucleotide sequences of the oligonucleotide primers used for RT–PCR are as follows: NOS-2 antisense (CTGCCATCTGGCATCTGGTA); NOS-2 sense (TAGAGGAACATCTGGCCAGGGTG); GAPDH antisense (ATGGCATGGACTGTGGTCAT); and GAPDH sense (ATGCCCCCATGTTTGTGATG).

TABLE 1. Nitrite Level in Supernatants of BCE Cells and Keratocytes Stimulated With Various Cytokines and LPS

	Nitrite (nmol/10^5 cells)	
Addition	*BCE Cells*	*Keratocytes*
None	0.10 ± 0.02	0.60 ± 0.04
LPS	0.15 ± 0.30	0.50 ± 0.02
IFNγ	0.05 ± 0.01	0.35 ± 0.15
TNFα	0.20 ± 0.10	0.50 ± 0.15
LPS + IFNγ	4.65 ± 0.85*	5.20 ± 0.75*
LPS + IFNγ + TNFα	7.25 ± 0.15*	7.95 ± 0.30*
IFNγ + TNFα	1.90 ± 0.10*	2.20 ± 0.45*
LPS + IFNγ + TNFα + IL-1β	7.30 ± 0.35*	8.15 ± 0.45*

BCE = basal cell epithelioma; LPS = lipopolysaccharides; IFNγ = interferon-γ; TNFα = tumor necrosis factor-α; IL-1β = interleukin-1β.
BCE cells and keratocytes were incubated with IFNγ (100 U/ml), IL-1β (100 U/ml), LPS (1 μg/ml), or TNFα (100 U/ml) alone or in combination. After 48 hours, nitrite levels were measured in the culture supernatants using the Griess reagent. Values are means ± SEM for four independent cultures, each done in duplicate.
* $P < 0.001$ vs untreated cultures.

Statistical Analysis

Results are expressed as mean ± SEM. Statistical analysis was performed with the nonparametric Mann–Whitney test. $P < 0.05$ was considered significant.

RESULTS

To determine whether NO production is inducible in BCE cells and keratocytes, confluent cell cultures were exposed to various stimuli for 48 hours. Mediators (LPS, IFN-γ, TNF-α and IL-1$_\beta$) were chosen for their capacity to induce NO biosynthesis in bovine RPE cells.[6] Generation of NO was measured as the accumulation of the stable end-product nitrite in the culture medium. When added separately, none of the mentioned stimulants could induce a significant production of nitrite by BCE cells and keratocytes (Table 1). Various combinations of stimulatory agents were tested to evaluate their effects on NO production (Table 1). In these conditions, a significant increase in NO biosynthesis was found when IFN-γ was associated with LPS. Further addition of TNF-α resulted in maximum nitrite release, greater than 10 μM/48 hours, whereas addition of IL-1$_\beta$ did not further increase nitrite release (Table 1).

The response to LPS, when added in conjunction with IFN-γ, was dose-dependent, with 50% of maximum nitrite accumulation observed at 0.1 μg/ml and 0.5 μg/ml in keratocytes (Fig. 1A) and in BCE cells, respectively (Fig. 2A). Maximum accumulation was observed at LPS concentrations averaging 1 μg/ml in both corneal cells. Furthermore, treatment of the cells with LPS in combination with different concentrations of IFN-γ induced keratocytes (Fig. 1B) and BCE cells (Fig. 2B) to produce nitrite in a dose-dependent manner. Optimal costimulation was achieved between 100 U/ml and 1000 U/ml IFN-γ. Figure 3 shows the time course of nitrite formation during stimulation with LPS, IFN-γ, and TNF-α. Nitrite production was first detected in keratocytes and in BCE cells after 12 hours of incubation, with a more rapid increase in keratocytes. In both cells, nitrite biosynthesis seemed to approach a plateau at 72 hours. A very similar kinetic was obtained with the stimulation with LPS and IFN-γ (data not shown).

Data given in Table 2 demonstrate that cytokine-induced nitrite release was markedly decreased by

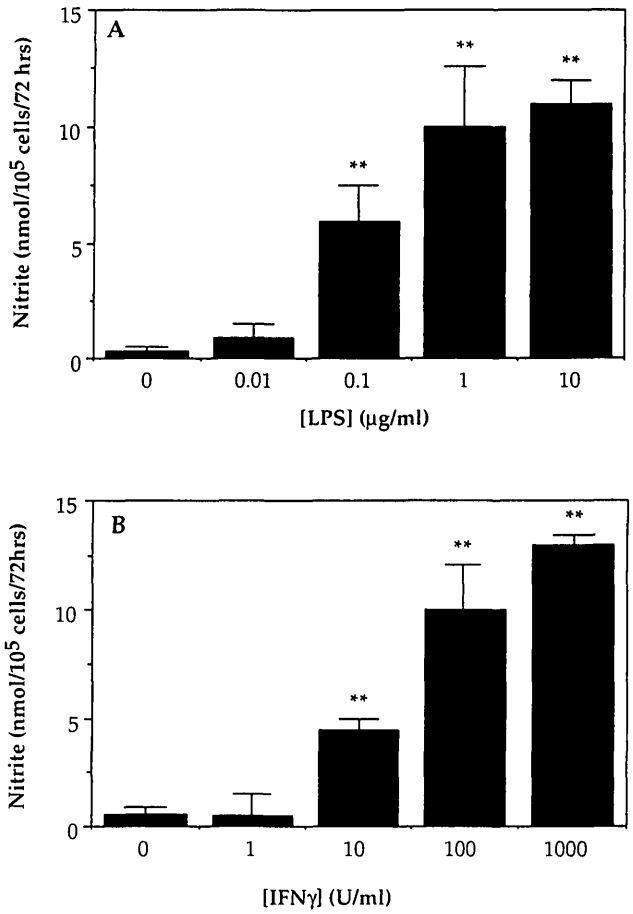

FIGURE 1. Effect of increasing lipopolysaccharide or interferon-γ concentrations on nitrite release from keratocytes. Cells were incubated with (**A**) 100 U/ml interferon-γ and different concentrations of lipopolysaccharide, or (**B**) with 1 μg/ml of lipopolysaccharide and different concentrations of interferon-γ. After 48 hours of incubation, the nitrite level was evaluated in the supernatants, as described in Materials and Methods. Values are means ± SEM for three independent cultures, each done in duplicate. **$P < 0.01$ when compared with data in controls.

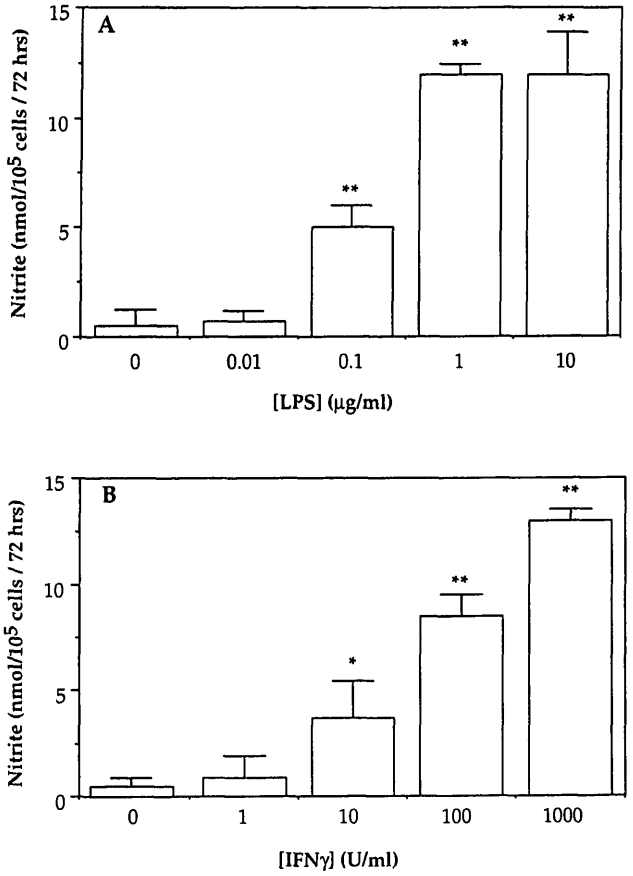

FIGURE 2. Effect of increasing lipopolysaccharide or interferon-γ concentrations on nitrite release from bovine corneal endothelial cells. Cells were incubated with (**A**) 100 U/ml interferon-γ and different concentrations of lipopolysaccharide, or (**B**) with 1 μg/ml of lipopolysaccharide and different concentrations of interferon-γ. After 72 hours of incubation, the nitrite level was evaluated in the supernatants, as described in Materials and Methods. Values are means ± SEM for three independent cultures, each done in duplicate. $*P < 0.05$ and $**P < 0.01$ when compared with data in controls.

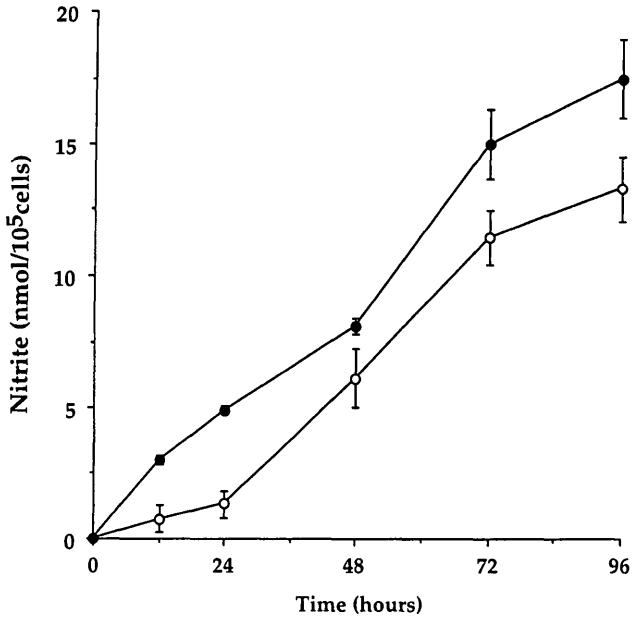

FIGURE 3. Time course of nitrite production in keratocytes and bovine corneal endothelial cells. Keratocytes (●) and bovine corneal endothelial cells (○) were incubated with lipopolysaccharide (1 μg/ml) plus interferon-γ (100 U/ml) and tumor necrosis factor-α (100 U/ml) in fresh Dulbecco's modified essential medium. Supernatants were harvested at indicated time points, and nitrite was determined using the Griess reagent, as described in Materials and Methods. Values are means ± SEM for two independent cultures, with each determination done in duplicate.

coincubation with an L-arginine analogue, N^G-monomethyl-L-arginine, confirming that nitrite production was related to NOS activity. Furthermore, addition of a more selective inhibitor for the inducible form (NOS-2), L-N^6-(1-Iminoethyl)lysine, also blocked the nitrite production caused by LPS–IFN-γ. The addition of cycloheximide also prevented nitrite accumulation (Table 2), demonstrating that protein synthesis is required for the production of NO.

Because basic FGF-2 and (TGF-β) are well described as regulators of NOS-2 induction in different cell types and particularly in retinal cells,[7,8,10,20] we tested their effects on LPS–IFN-γ-induced nitrite release. Coincubation of keratocytes cells with FGF-2 and NOS inducers (LPS–IFN-γ) for 72 hours markedly reduced nitrite production, whereas the addition of TGF-β significantly enhanced cytokine-induced nitrite release (Fig. 4A). In BCE cells, a similar potentiation of LPS–IFN-γ-induced nitrite release was obtained with TGF-β, but no significant inhibition could be observed with FGF-2 (Fig. 4B).

To determine whether nitrite release by activated keratocytes and BCE cells was related to NOS-2 mRNA expression, RNA was extracted from these cells after 24 hours of stimulation with different combinations

TABLE 2. Inhibition of Cytokine-Induced Nitrite Formation by L-Arginine Analogues and Cycloheximide

	Nitrite (nmol/10⁵ cells)	
	BCE Cells	*Keratocytes*
Control	6.1 ± 0.7	8.2 ± 0.5
L-NMMA (0.1 mM)	1.5 ± 0.1*	1.7 ± 0.4*
L-NIL (0.1 mM)	1.5 ± 0.8	1.4 ± 0.1*
Cycloheximide (0.25 μg/ml)	2.0 ± 0.3	3.8 ± 0.5*

Cells were treated with LPS (1 μg/ml), IFNγ (100 U/ml), and TNFα (100 U/ml) without (control) or with the indicated agents for 48 hours. After this time, the nitrite release in the supernatants was performed. Values are means ± SEM for three independent cultures, each done in duplicate.
* $P < 0.001$ vs control.

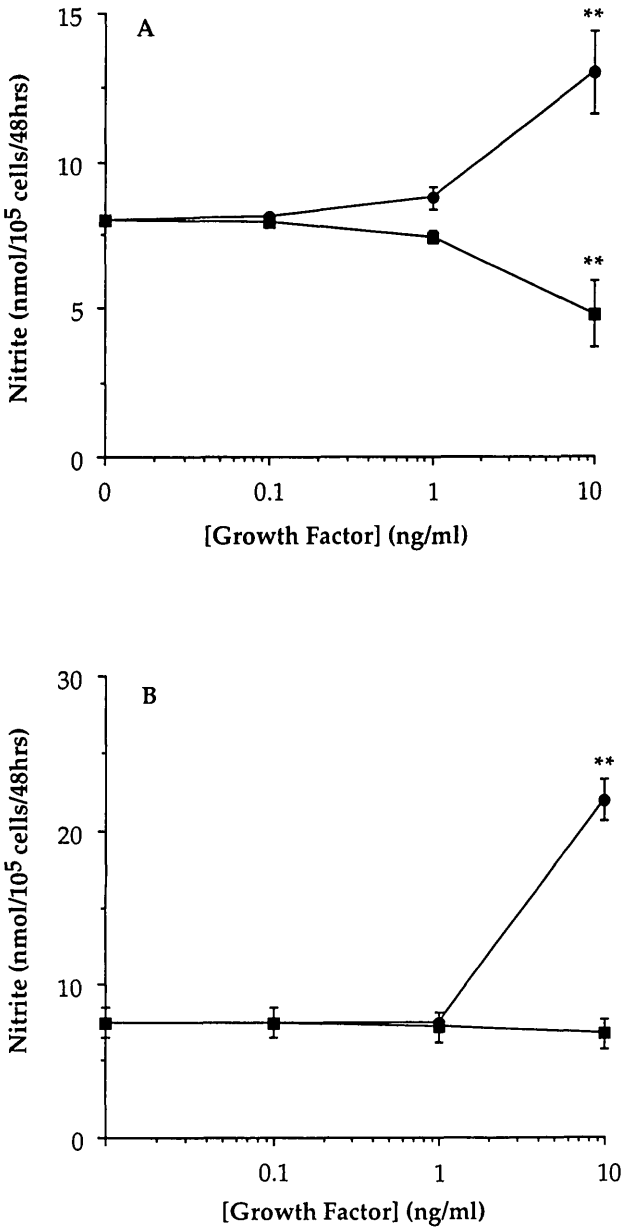

FIGURE 4. Effect of increasing concentrations of transforming growth factor-β and fibroblast growth factor-2 on lipopolysaccharide–interferon-γ-induced nitrite release from (**A**) keratocytes and (**B**) bovine corneal endothelial cells. Keratocytes and bovine corneal endothelial cells were costimulated with interferon-γ (100 U/ml), lipopolysaccharide (1 μg/ml), and indicated concentrations of transforming growth factor-β (•) or fibroblast growth factor-2 (■). After 48 hours, culture media were assayed for nitrite. Values are means ± SEM for three experiments, each done in duplicate. $^{**}P < 0.01$ when compared with data in controls.

of LPS, IFN-γ and growth factors and then was examined by RT–PCR. A specific RT–PCR product for NOS-2 at the predicted size of 350 bp was seen on agarose gel electrophoresis after amplification of complementary DNA (cDNA) from LPS–IFN-γ-stimulated keratocytes (Fig. 5A) and BCE cells (Fig. 5B), using NOS-2-specific primers. The NOS-2 RT–PCR product was absent in RNA from unstimulated keratocytes and BCE cells (Fig. 5, lane 1). In keratocytes, IFN-γ alone was able to induce NOS-2 mRNA (Fig. 5A, lane 3), but to a lesser extent than the combination LPS–IFN-γ (Fig. 5A, lane 4), whereas LPS alone had no effect on NOS-2 mRNA accumulation (Fig. 5A, lane 2). In these cells, the addition of FGF-2 in the culture medium inhibited the accumulation of NOS-2 mRNA in keratocytes stimulated with LPS and IFN-γ (Fig. 5A, lane 6). However, we were unable to detect a change in the accumulation of NOS-2 mRNA when TGF-β was present with LPS–IFN-γ (Fig. 5A, lane 5), suggesting that the increase of TGF-β on LPS–IFN-γ-induced nitrite release did not operate at the mRNA level. The situation was different in BCE cells: A faint band could be detected with LPS alone (Fig. 5B, lane 2) and a more prominent band with IFN-γ alone (Fig. 5B, lane 3). The maximum induction was obtained with costimulation LPS–IFN-γ (Fig. 5B, lane 4), as was true in keratocytes. Concerning the effects of the two growth factors, we could not detect differences in NOS-2 mRNA accumulation with TGF-β (Fig. 5B, lane 5) or with FGF-2 (Fig. 5B, lane 6). No increase in the intensity of NOS-2 mRNA could be observed when TNF-α was added with LPS–IFN-γ in both corneal cell types (Fig. 5, lanes 7).

DISCUSSION

In this article, our results demonstrate that BCE cells and keratocytes are able to produce nitrite with cytokine and LPS stimulation. The maximum production was observed with the combination of LPS, IFN-γ, and TNF-α. The inhibition of cytokine-induced nitrite release by L-arginine analogues N^G-monomethyl-L-arginine and L-N^6-(1-Iminoethyl)lysine demonstrates that nitrite production results from the oxidation of L-arginine through the NOS pathway. The inhibitory effects of cycloheximide indicate that IFN-γ and LPS induce the synthesis of a protein that is required for the production of NO by these cells. Analysis by RT–PCR demonstrates the induction of NOS-2, the specific inducible form of NOS, after LPS and IFN-γ treatment in both cell types. We demonstrate by this technique that IFN-γ alone can induce NOS-2 mRNA, whereas a significant nitrite release could be detected in the culture medium of BCE and keratocytes cells only after costimulation with LPS. The explanation for this result could be that in the presence of IFN-γ alone, the small level of NOS-2 mRNA could not be translated or could be rapidly destabilized, and that the presence of LPS is required for NOS-2 mRNA translation or for increasing the stability of the messenger, as previously described in macrophages.[21] It is likely that LPS, IFN-γ, or TNF-α as individual agents

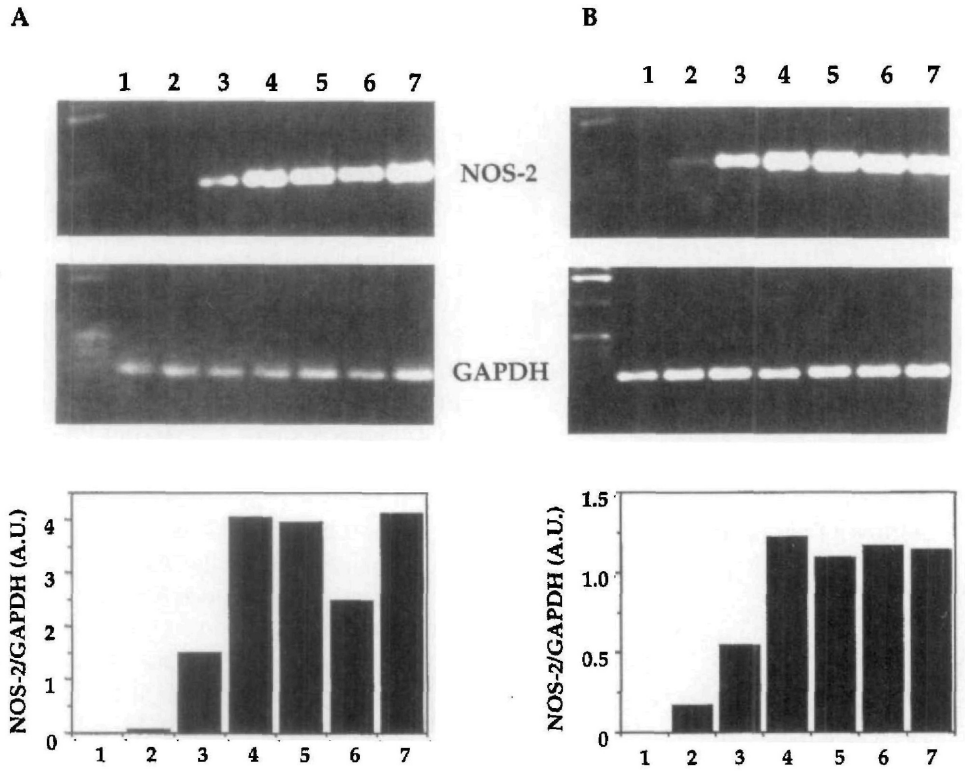

FIGURE 5. Regulation of nitric oxide synthase-2 messenger RNA accumulation in keratocytes and bovine corneal endothelial cells. Total RNA from (**A**) keratocytes and (**B**) bovine corneal endothelial cells was isolated 24 hours after different treatments: (1) control, (2) lipopolysaccharide, (3) interferon-γ, (4) lipopolysaccharide–interferon-γ, (5) lipopolysaccharide–interferon-γ + transforming growth factor-β, (6) lipopolysaccharide–interferon-γ + fibroblast growth factor-2, (7) lipopolysaccharide–interferon-γ + tumor necrosis factor-α. The levels of nitric oxide synthase-2 and reduced glyceraldehyde–phosphate dehydrogenase messenger RNA were assessed by RT–PCR analysis as described in Materials and Methods. The experiment represents one of three independent trials that yielded similar results.

have no effect on nitrite production, because they are not able to induce intracellular signals sufficient for a full transcription or translation of the NOS-2 gene.

Our results further demonstrate that NOS-2 mRNA accumulation and subsequent nitrite release by activated keratocytes could be modified by the presence of growth factors. Fibroblast growth factor-2 reduced LPS–IFN-γ-induced nitrite production and NOS-2 mRNA accumulation, as in bovine RPE cells,[20,22] and TGF-β increased nitrite production, as it does in bovine RPE cells.[20] In RT–PCR analysis, we could not detect differences in the level of NOS-2 mRNA. This result contrasts with the increase of NOS-2 mRNA by TGF-β reported in Swiss 3T3 fibroblasts[23] and chondrocytes.[24] It could be hypothesized that TGF-β acts at a posttranscriptional level. An indirect mechanism could also operate—an effect on the metabolism of L-arginine (a modulation of arginase activity) or as an effect of the metabolism of tetrahydrobiopterin (BH_4), an indispensable cofactor for NOS activity.[1–4] In this case, a positive effect of TGF-β on GTP-cyclohydrolase-I (GTP-CH-I), the rate-limiting enzyme in the BH_4 synthesis, could increase the intracellular level of BH_4 and then exacerbate NOS activity. In that we know that this enzyme was induced by LPS in a different model,[25,26] it would be interesting to study the effect of TGF-β on the induction of the GTP-cyclohydrolase-I mRNA in corneal cells.

The situation is different in BCE cells, because FGF-2 had no effect on NOS-2 mRNA level and NO production, even though TGF-β potentiated the nitrite release but not the accumulation of NOS-2 mRNA. These results reinforce the fact that the modulatory effect of the growth factors on NOS-2 induction and NO synthesis is largely dependent on the type of cells used and the type of growth factor involved,[3,4] as has been described in RPE cells.[7,8,20]

Recent data support an important role for cytokines in corneal disease[27] as a factor in infections, in dystrophy, and in corneal graft rejection.[17,28–31] Because in inflammation of the anterior segment of the eye ocular cells are associated with cytokine production, NOS-2 must be induced in corneal cells. Additional studies will be required to determine the bal-

ance between the beneficial properties of NO (antimicrobial and antiviral activities)[4,32] and NO's potential tissue-damaging effects. Nitric oxide could exert a noxious effect on the corneal cells themselves. As described in the proximal tubule epithelium,[33] NO could inhibit the endothelial Na^+/K^+-ATPase. The active transport of ions from the stroma into the aqueous, induces a secondary passive movement of water. This endothelial "fluid pump" is responsible in large part for corneal transparency. Therefore, if NO disturbed the endothelial pump, corneal transparency could be lost. Furthermore, NO could modulate the synthesis of extracellular matrix proteins, as reported in mesangial cells.[34] This phenomenon could also play an important role in corneal wound healing.

Corneal endothelial cells and keratocytes have been demonstrated to express NOS-2 and release NO when stimulated by cytokines and LPS; therefore, NO could be implicated in corneal inflammatory diseases. The precise role of NO in the pathogenesis of corneal inflammation, corneal transparency, and corneal wound healing remains to be determined.

Key Words

cell culture, cornea, cytokines, nitric oxide

Acknowledgments

The authors thank Hervé Coet for photographic work.

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