Regulation of Nitric Oxide Synthase 2 in Rabbit Corneal Cells

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PURPOSE. The purpose of these studies was to investigate the role of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin 1β (IL-1β), and transforming growth factor-β (TGF-β) in the regulation of inducible nitric oxide synthase (NOS2) activity in rabbit corneal cells.

METHODS. Rabbit corneal epithelial, stromal, and endothelial cells were grown in culture and treated with cytokines and growth factors, alone or in combination. NOS activity was measured at times up to 72 hours after treatment by assaying the culture medium for nitrite using the Griess reaction. Cell lysates were analyzed by Western blot analysis for NOS2 protein. RNA was isolated and amplified with NOS1-, NOS2-, and NOS3-specific primers by RT-PCR.

RESULTS. NOS2 expression was induced by combined cytokine treatment from nondetectable levels to abundant levels in low passage (<4) stromal cells and to low levels in corneal endothelial cells but not in corneal epithelial cells. In the absence of IFN-γ, little or no nitrite accumulation was induced by TNF-α, IL-1β, or lipopolysaccharide (LPS) treatment. The inductive effects of IFN-γ were antagonized in a dose-dependent manner by the myxoma virus rabbit IFN-γ receptor homolog, M-γ. rRafIFN-γ, in combination with IL-1β and TNF-α, induced the appearance of NOS2 mRNA within 24 hours but detectable nitrite did not accumulate in large amounts (>10 μM) until after 24 hours postinduction. NOS2 was identified as a 130 kDa protein on Western blot analysis using monoclonal antibody against murine NOS2. TGF-β and β2 inhibited the accumulation of cytokine-induced nitrite in a dose-dependent manner while not significantly reducing the steady state level of NOS2 mRNA. The activity of the induced NOS was inhibited by 1400W, a NOS2-selective inhibitor, but not 7-nitroindazole, a NOS1-selective inhibitor.

CONCLUSIONS. In cultured corneal stromal cells, NOS2 expression was upregulated by IFN-γ in combination with IL-1β and TNF-α but not by any of these cytokines alone, while TGF-β downregulated the activity. Cultures of corneal epithelial cells could not be induced to express NOS2, yet cultures of endothelial cells produced low amounts of NO in response to cytokines. The NOS1 and NOS3 isoforms were not detected in any of these corneal cells. (Invest Ophthalmol Vis Sci. 2001;42:713–719)

Nitric oxide (NO), generated by three isoforms of nitric oxide synthase (NOS), is believed to play diverse roles in the eye.1,2 NOS enzymes convert arginine to citrulline, releasing the highly reactive free radical NO. NOS1 (bNOS), an isoform constitutively expressed principally in neurons and cells associated with neurons, is suspected to produce low amounts of NO, which functions as a neurotransmitter in the retina.3 NOS2 (iNOS), an inducible form of the enzyme present in macrophages and some other cell types, is believed to contribute to the pathogenesis of endotoxin-induced uveitis and possibly glaucoma.4–6 NOS3 (eNOS), a second constitutively expressed isoform found principally in vascular endothelial cells, may contribute to the breakdown of the blood/aqueous barrier and/or outflow of aqueous humor.7,8 In rare circumstances, NOS1 and NOS3 can be inducible, while NOS2 expression can be constitutive; thus, cell type and stimulus-dependent regulation must be examined on a cell-by-cell basis.9

NOS2 is considered part of the innate immune response.10,11 It differs from the other two isoforms in that it is inducibly regulated by cytokines, its activity is not highly calcium-dependent, and the amount of NO it produces (as measured by turnover number) is three or more times greater than other isoforms.12 Thus, the relatively large amounts of NO and its reaction products produced by NOS2 are capable of killing bacteria, viruses, and other infectious organisms and are also capable of causing tissue damage.13 NO is extremely reactive and short-lived. A variety of reactive products of NO formed in tissues, including peroxynitrite, NOX, and N2O3, are likely the molecules responsible for tissue damage.12–14

The role of NO in the pathogenesis of inflammatory eye diseases is not well understood. The cells responsible for its production and the factors that regulate the expression of NOS and its activity are areas of considerable interest. We are interested in the role that NO may play in producing corneal edema. The mechanisms responsible for corneal edema associated with infection and inflammation may have several important mediators, but NO could be a contributing factor.15–19 For example, it is clear that alterations in corneal endothelial cell pump activity occur during inflammatory edema due to HSV infection, but direct identification of the effector has been difficult to establish. It has been proposed that NO may play a role in producing corneal edema associated with uveitis10,17 and it has been documented that NO inhibitor therapy can reduce the severity of edema.19 On the other hand, NO produced by NOS3 has been reported to be a positive effector of corneal detergence, but it is unclear how this enzyme and its products function to regulate corneal hydration.18

The work presented here focuses on the regulation of NOS expression in cultured rabbit corneal cells, documenting that inflammatory cytokines upregulate NOS2 expression in corneal stromal and endothelial cells in the absence of detectable NOS1 or NOS3. IFN-γ and its receptor are shown to be required for NOS2 gene expression and NO production, while TGF-β inhibits NO2 accumulation. The induction of NOS2 expression in corneal cells by proinflammatory cytokines makes NO a potential regulator of corneal hydration during anterior segment inflammation.
METHODS

Cell Culture

Rabbit corneal epithelial, stromal and endothelial cells were isolated, grown to confluence, and subcultured with slight modifications to published procedures. Primary cultures were established from New Zealand white rabbits of either sex. Rabbits of either sex were obtained from a local supplier and determined to be free of defects by slit-lamp examination. The animals were housed in an animal quarters that was approved by the American Association for Laboratory Animal Science and handled and killed in accordance with the policies stated in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Corneal epithelial cells were isolated by Dispase II (Boehringer Mannheim, Mannheim, Germany) digestion of the anterior portions of excised corneas after removal of the epithelium and posterior stroma.20 After Dispase II digestion at 4°C for 16 hours, the epithelium was removed by gentle scraping and digested to single cell suspension by treatment with trypsin/EDTA. Trypsin digestion was terminated with Type I-S soybean trypsin inhibitor (Sigma, St. Louis, MO), and the cells harvested by centrifugation. Epithelial cells recovered from a single cornea were seeded into 25 cm² Primaria flasks (Becton Dickinson, Lincoln Park, NJ) and grown to confluence at 34°C in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/l glucose, 5% heat-inactivated defined fetal bovine serum, 100 mM sodium pyruvate, 1% non-essential amino acids, 1% antibiotic/antimycotic (Gibco BRL, Gaithersburg, MD), and 1% L-glutamine, 1% sodium bicarbonate, 1% sodium selenite, 1.5 mM EDTA, 0.2 mM phenylmethylsulfonylfluoride, 7 mM β-mercaptoethanol (Bayer Corporation, Kankakee, IL). Cells were passaged at a 1:2 split ratio using 0.04% trypsin and 0.1% EDTA. Cells were used in the first 3 passages. Corneal endothelial cells were isolated from Descemet's membrane by digestion with 1 unit/ml Dispase II for 40 minutes at 37°C in calcium- and magnesium-free HBSS. Cells were harvested by centrifugation, seeded in Costar plastic tissue cultureware (Corning Inc., Corning, NY) and grown to confluence in high-glucose DMEM containing 5 µg/ml gentamicin. Corneal stromal cells were isolated from excised corneas after removal of the epithelium and endothelium by scraping.21 The stroma was digested in 150 units/ml collagenase (Clostridium histolyticum, Life Technologies, Grand Island, NY) containing 5 µg/ml gentamicin. Corneal stromal cells were isolated by gentle scraping and digested to single cell suspension by trypsin/EDTA. The cells were recovered by centrifugation, suspended in growth medium, and grown to confluence at 34°C in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/l glucose, 5% heat-inactivated defined fetal bovine serum (FBS; HyClone, Logan, UT), 0.01% Mito + serum extender (Collaborative Biomedical Products, Bedford, MA), and 10 µg/ml ciprofloxacin (Bayer Corporation, Kankakee, IL). Cells were passaged at a 1:2 split ratio using 0.04% trypsin and 0.1% EDTA. Cells were used in the first 3 passages. Corneal endothelial cells were isolated from Descemet's membrane by digestion with 1 unit/ml Dispase II for 40 minutes at 37°C in calcium- and magnesium-free HBSS. Cells were harvested by centrifugation, seeded in Costar plastic tissue cultureware (Corning Inc., Corning, NY) and grown to confluence in high-glucose DMEM containing 5% FBS, 0.01% Mito +, and 10 µg/ml ciprofloxacin. All cells were routinely checked for mycoplasma contamination by Hoechst stain.

Recombinant Rabbit Interferon-γ, Myxoma Virus Interferon Receptor Homolog/Antagonist, and Cytokines

Recombinant rabbit IFN-γ (rRIFN-γ) was produced by subcloning the rabbit IFN-γ gene obtained from plasmid pBR322 (pR7exp; Genentech Inc., San Francisco, CA) into a PET-NB4 plasmid (obtained from Kent Wilcox, Medical College of Wisconsin, Milwaukee, WI). The IFN-γ gene was fused with an N-terminal histidine tag and placed under control of the lac operator/repressor so that production was induced by isopropylthiogalactoside (IPTG). The IFN-γ histidine fusion protein was isolated from lysates of transformed cultures of Escherichia coli DL21 (DE3) using cobalt affinity columns (Clontech Laboratories, Palo Alto, CA). The purified IFN-γ produced a single band of approximately 12 kDa on SDS PAGE. The purified protein contained no detectable endotoxin at the dilutions used to induce NO as determined by E-TOXATE test (<0.05 units; Sigma). Purified M-γ protein, a viral interferon-γ receptor homolog from myxoma virus-infected cell supernatants, was purified as described previously.22 Other cytokines included recombinant murine tumor necrosis factor-α (TNF-α), recombinant human interleukin 1β (IL-1β), and recombinant human transforming growth factor β1 and β2 (TGF-β1 and TGF-β2, R and D Systems, Inc., Minneapolis, MN). Lipopolysaccharide (LPS) prepared from Salmonella typhimurium was obtained from Sigma.

Reverse Transcriptase–Polymerase Chain Reaction

Cultures of cells were treated with Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's instructions to extract RNA and DNA. The isolated RNA was reverse transcribed using MuLV reverse transcriptase (Applied Biosystems, Foster City, CA) and primed with either random hexamers (Applied Biosystems) or the 3′-specific NO2 primer at 42°C for 2 hours. The sequences of primers used for the three NOS isoforms are shown in Table 1. The primers for NO1 and NO3 were provided by John Baker (Medical College of Wisconsin).23 Primers for rabbit glyceraldehyde-3-phosphate dehydrogenase were as previously described. The reverse transcriptase reaction mixture contained 1 µg total RNA, 5 mM MgCl2, 1 mM dNTPs, 1 units/µl RNase inhibitor, 4 units/µl MuLV reverse transcriptase (Applied Biosystems), 2.5 µM random hexamers, or 3′-specific NO2 primer. The cDNA was amplified using AmpliTag Gold (Applied Biosystems). Approximately 10% of the RT reaction mixture was used in each PCR reaction containing 1 mM MgCl2, 250 µM each dNTPs, 0.2 to 0.4 µM primers and 2.5 units AmpliTaq Gold in reaction buffer (10 mM Tris-HCl pH 8.3 and 50 mM KCl). The PCR reaction cycle was 95°C for 10 minutes then 40 cycles of 94°C for 1 minute, 62°C for 1 minute, and 72°C for 1 minute (Perkin-Elmer 480). The reaction products were detected on ethidium bromide-stained agarose gels. Reaction products were identified after Southern blotting and hybridization to 32P-labeled probes.

Western Blot Analysis

Cultures of cells were rinsed with HBBS and lysed directly in lysis buffer (10% glycerol, 50 mM Tris-HCl, pH 7.0, 2% sodium dodecyl sulfate, 1.5 mM EDTA, 0.2 mM phenylmethylsulfonylfluoride, 7 mM β-mercaptoethanol, 0.2 mg aprotinin per ml, 50 mM bromophenol blue). Equal amounts of total protein were subjected to SDS PAGE. Immuno-bLOTS were prepared by electrophoretic transfer and probed with monoclonal antibody to mouse NO2 (Transduction Laboratories, Lexington, KY). This antibody recognizes an epitope in the NADPH binding region of NO2 and therefore binds NO2 of several species. Protein concentration was determined by the Pierce BCA Protein Assay (Pierce, Rockford, IL).

Nitrate Detection

NO is unstable in culture medium. Nitrite, a stable product of NO production, was used as a measure of NO activity and was detected using the Griess reaction.25

Statistical Analysis

Means were compared by one-way analysis of variance and the significance of differences among means of treatment groups were deter-
RESULTS

Cytokine-Induced NOS Activity in Cultured Corneal Cells

Primary cultures of rabbit corneal epithelial, stromal, and endothelial cells were grown to confluence and treated with mixtures of proinflammatory cytokines, rRαIFN-γ, TNF-α, and IL-1β. After exposure to cytokines for 48 hours to 72 hours, nitrite accumulation in the growth medium was measured and the cells lysed to extract RNA for RT-PCR analysis. In confluent primary cultures of corneal epithelial cells receiving all three cytokines, low amounts of nitrates accumulated by 48 hours of 19 ± 0.17 μM (n = 4), while at 72 hours only 0.48 ± 0.09 μM (n = 3) nitrate per 5.0 × 10^6 cells in excess of controls receiving no cytokines. Using RT-PCR to detect mRNAs of each of the three isoforms of NOS, it was determined that mRNA for NOS1, NOS2, and NOS3 could not be detected.

In contrast, stromal cell NOS2 appears to be strongly induced by the mixture of the three cytokines. Primary cultures of stromal cells from three individual corneas induced by rRαIFN-γ, TNF-α, and IL-1β accumulated 19.5 ± 7.7 μM nitrite in excess of untreated controls after 72 hours. Cultures of stromal cells were used after no more than four passages in culture. Cultures in passages 1 through 3 established from 10 eyes of 10 different rabbits produced a net increase in medium nitrite concentration of 13.2 ± 3.0 μM/1.0 × 10^6 cells (n = 3) after 48 hours, a significant increase relative to controls receiving no cytokines (2.0 ± 0.3 μM/1.0 × 10^6 cells; P < 0.05). RT-PCR documented the abundant expression of NOS2 mRNA. Neither NOS1 nor NOS3 mRNA was detectable. The range of nitrite accumulation in cytokine-treated cultures in excess of untreated controls was 5.8 μM to 51.5 μM, depending on the age and passage of the culture. Lysates of stromal cell cultures and preparations of cell-free enzyme extracts converted arginine to citrulline in a time-dependent and protein-dependent manner that was not dependent on added Ca or calmodulin but was inhibited by 2 mM N^ε-methyl-L-arginine, a specific inhibitor of NOS (data not shown). These observations document that cytokines induce NOS2 expression in cultured corneal stromal cells.

Cultures of rabbit corneal endothelial cells treated with mixtures of the cytokines TNF-α, IL-1β and rRαIFN-γ produced a net accumulation of 0.64 ± 0.50 μM nitrite/10^6 cells (n = 6) after 48 hours and 1.30 ± 1.1 μM nitrite/10^6 cells (n = 3) after 72 hours, relative to controls that were not treated with cytokines. These amounts were more than 10-fold lower than those produced by stromal cell cultures. RT-PCR could detect NOS2 in RNA prepared from endothelial cells but neither NOS1 nor NOS3 could be detected.

Induction of NOS2 Expression by Cytokines in Rabbit Corneal Stromal Cells

The ability of cells and tissues to regulate the production and accumulation of NO is critical. The production of NO by constitutively expressed low output NOS1 and NOS3 is regulated primarily at the level of enzyme activity. 26 NOS2, an enzyme capable of producing large amounts of nitrite (accumulations of 10 μM or greater), is primarily regulated at the transcriptional level. 13 Proinflammatory cytokines and bacterial lipopolysaccharides (LPS) appear to be potent upregulators of NOS2 gene expression, but as studies on regulation of NOS2 in various cell types and tissues have emerged, it has become clear that the specific effects of these regulators are cell type- and species-dependent. 13

The combined effects of the proinflammatory cytokines TNF-α, IL-1β, and rRαIFN-γ, as well as LPS, on NOS2 expression and NO accumulation in rabbit corneal stromal cells are significant (Fig. 1A). At the concentrations of cytokines used in these studies, none of the cytokines were effective inducers when used alone. Western blot analysis clearly demonstrated that those cultures which have significantly increased nitrite also showed evidence of the 130 kDa NOS2 protein (Fig. 1B). RT-PCR, the most sensitive of the three methods that we have used, confirms that NOS2 mRNA was induced in cultures treated with the mixture of rRα IFN-γ, TNF-α, and IL-1β, which expressed significantly increased nitrite (Fig. 1C). Messenger RNAs of NOS1 and NOS3 remained undetectable.

The use of isoform-selective inhibitors of NOS helped confirm the isoform responsible for the production of NO. Isoform-selective inhibitors of NOS are rare. 27,28 The N-(3-aminomethyl)benzyl)acetamide, a compound referred to as 1400 W, is one of the most NOS2-selective inhibitors available, being 200- and 5000-fold more effective in inhibiting NOS2 than NOS1 and NOS3, respectively. 28 The compound 1400 W produced a dose-dependent inhibition of cytokine-inducible nitrite accumulation in rabbit corneal stromal cell cultures, resulting in 50% inhibition of nitrite accumulation at approximately 5 μM (Fig. 2). The potent in vitro inhibitor of NOS1 and NOS3,
7-nitroindazole, produced less than 25% inhibition of cytokine-induced NOS activity in stromal cells at 50 μM. These results further confirmed that the cytokines IFN-γ, TNF-α, and IL-1β induced NOS activity with the characteristics of NOS2 in cultured rabbit corneal stromal cells.

**Time Course of Induction of NOS Activity**

The regulation of NOS2 activity is complicated, thus it is necessary to study each step from signal transduction to the production of NO from arginine. Our data document that combinations of IL-1β, TNF-α, and rRaIFN-γ were required for production of NOS2 mRNA and protein resulting in accumulation of nitrite. The kinetics of the induction process appeared slow based on the accumulation of nitrite, which required 24 to 36 hours after initial cytokine treatment to achieve a significant rise above background levels (Fig. 3A). Nitrites continue to accumulate to highly significant levels from 24 to 72 hours postinduction. RT-PCR indicated that NOS2 mRNA becomes detectable 24 to 36 hours after initiation of cytokine treatment of stromal cells (Fig. 3C). The detection of the NOS2 protein reached detectable levels by 36 hours and continued to increase in amount to 72 hours (Fig. 3B). Thus, in rabbit corneal stromal cells, the measurement of nitrite accumulation represented the production of functional NOS2 protein.

**Dependence of NOS2 Induction on Dose of IFN-γ and IFN-γ Receptor**

Whether individually or combined, the inflammatory cytokines IL-1β and TNF-α did not induce NOS2 expression in the absence of rRaIFN-γ (Fig. 1A). The regulation of NOS2 expression by sequences present in the 5′ untranslated region of the gene is important in controlling NO production. It has been hypothesized that the IFN-γ receptor's ability to induce NOS2, as it does with other mediators of inflammation. IFN-γ is hypothesized to exert its effects through the induction of IRF-1, an IFN-γ-induced transcription factor that is capable of binding to an IκB response element (IRE) in the NOS2 promoter. The ability of rRaIFN-γ to enhance the induction of nitrite accumulation in rabbit corneal stromal cells was dose-dependent, reaching a point of saturation and thus suggesting receptor function (Fig. 4A).

The induction of nitrite accumulation by IFN-γ was species-specific. To test the species-specificity of IFN-γ’s ability to induce NOS2, we treated triplicate cultures of confluent low passage (≤ 3) rabbit corneal stromal cells with recombinant...
human IFN-γ alone or in combination with TNF-α and IL-1β. Forty-eight hours after initiation of cytokine treatment, the media were sampled to detect nitrates, the cells harvested, and RNA extracted for RT-PCR. The level of nitrite in untreated controls was 2.4 ± 0.3 μM, which was not significantly different from cultures treated with rHuIFN-γ alone (100 units/ml; 2.7 ± 0.3 μM) or cells treated with the cytokine mixture (2.8 ± 0.3 μM). These levels of nitrite were all significantly less than in those cultures treated with TNF-α, IL-1β, and rRaIFN-γ (15.7 ± 0.4 μM, P < 0.05). Increasing the dose of rHuIFN-γ to 100 to 1000 units/ml in combination with TNF-α and IL-1β did not result in significant nitrite accumulation or detectable mRNA production. These observations suggest that rRaIFN-γ exerted its affects through receptor-mediated signal transduction.

In an effort to ensure that the effects of rRaIFN-γ were due to interaction with its receptor, we tested the ability of RaIFN-γ receptor homolog, M-17, to inhibit the effects of rRaIFN-γ. Rabbit stromal cells were treated with an inducing dose of IL-1β, TNF-α, and rRaIFN-γ. Varying amounts of purified myxoma virus IFN-γ receptor homolog were added to compete with the stromal cell IFN-γ receptor for limited amounts of rRaIFN-γ. The homolog effectively competed out the effect of the rRaIFN-γ, resulting in dose-dependent inhibition of nitrite production by the receptor homolog (Fig. 4B). The inclusion of heparan sulfate with the receptor homolog did not alter its ability to reduce the production of nitrite, thus ensuring that the observed effects were IFN-γ receptor-dependent.

Inhibition of Nitrite Accumulation by Transforming Growth Factor

Cytokines and growth factors not only induce NOS2 but also regulate the accumulation of the nitrites it produces. The effects are species-, cell type-, and tissue type-dependent. In some cells, the regulatory effects of TGF-β on NOS occur by transcriptional mechanisms, yet in other cells the effects appear posttranslational. It has been reported that TGF-β increases the accumulation of nitrites in cultures of bovine corneal stromal cells. This observation was surprising in that TGF-β downregulates nitrite accumulation in most other cell types and tissues. Cultures of rabbit corneal stromal cells treated with NOS2 inducing mixtures of IL-1β, TNF-α, and rRaIFN-γ and varying concentrations of TGF-β, accumulated less nitrate than control cultures receiving no TGF-β (Fig. 5A). TGF-β, also decreased nitrate accumulation in a similar dose-dependent manner (data not shown). The steady state levels of NOS2 mRNA remained unchanged, despite reduced accumulation of NOS, suggesting that TGF-β exerts its effects by a posttranscriptional mechanism (Fig. 5C). Western blot analysis clearly documented a reduction of NOS2 protein in cells treated with increasing doses of TGF-β, suggesting inhibition at the level of translation or reduced protein stability (Fig. 5B).

**DISCUSSION**

Our data document NOS2 transcription, the production of NOS2 protein, and nitrite accumulation in cytokine-treated rabbit corneal stromal cells cultured for 4 passages or less. Western blot analysis prepared using a NOS2-specific monoclonal antibody demonstrated the appearance of a 130 kDa band in cytokine-treated rabbit stromal cells at times after the appearance of NOS2 mRNA. The appearance of the protein was coincident in time with the accumulation of nitrates. The observation of the NOS2 protein in combination with the ability to measure the conversion of arginine to citrulline in cell-free enzyme extracts prepared from cytokine-treated cultures confirmed the induction of NOS2. The ability to inhibit nitrite accumulation by greater than 50% with 5 μM 1400W, a highly NOS2-selective inhibitor, while achieving less than 24% inhibition by 7-nitroindazole, a NOS1 and NOS3-selective inhibitor in vitro, strongly supports the identity of the NOS activity as being that of NOS2.

The ability to induce NOS2 expression in rabbit corneal stromal cells required the presence of a mixture of three cytokines. Proinflammatory cytokines TNF-α, IL-1β, and IFN-γ were required to induce significant nitrite accumulation, NOS2 mRNA accumulation, and NOS2 protein production. Unlike bovine corneal stromal cells, neither mixtures of LPS and IFN-γ nor TNF-α and IFN-γ induced significant nitrite production or provided evidence of NOS2 upregulation. Also, unlike cultured rat hepatocytes or rabbit articular chondrocytes, neither IL-1β nor TNF-α alone or in any combination induced NOS2.

The reasons for the difference in regulation of NOS2 expression among species is likely related to the 5′ and 3′ UTRs of the NOS2 gene. The coding sequence of the cDNAs for rats, mice, and humans share 80% to 90% homology; thus, the enzymes are quite similar. The 5′ UTR of rats and humans are substantially different, which likely accounts for the differences in the ability of cytokines to regulate gene expression. The 5′ proximal 1.5 Kbp sequences of the rat provide sufficient promoter activity to account for the full induction and pattern of cytokine responsiveness of NOS2 expression. This region includes binding sites for 20 or more transcription factors. The human NOS2 gene, however, is regulated by cytokine responsive elements contained within 16 Kb of the transcription start site. This region is only approximately 66% homologous to that of mice. It also appears that various cell types
within a given species possess unique cell type-specific mechanisms for effecting mRNA stability, in addition using alternative splicing and/or alternative transcription start sites. Thus, it is not unexpected to see the regulatory region of the NOS2 gene of rabbits to be different from that of mice and/or humans.

In all species examined to date, IFN-γ plays a critical role in regulating NOS2 expression. IFN-γ appears to function as an enhancer in most cell types, thus acting synergistically with other cytokines, yet in activated peritoneal mouse macrophages it may directly induce NOS upregulation.30,37 Our data document that rAlIFN-γ alone does not significantly induce NOS2 expression. rlIFN-γ, when combined with fixed doses of TNF-α and IL-1β, produces a dose-dependent but saturable induction of nitrite accumulation. These observations strongly implicate the IFN-γ receptor as an essential part of the induction mechanism. We have demonstrated that the IFN-γ receptor homolog of myxoma virus can effectively compete out the IFN-γ effect in a dose-dependent manner, further implicating the need for IFN-γ receptor function. Thus, in some cell types of rabbits, humans, bovines, mice, and rats, IFN-γ is absolutely required to produce large amounts of nitrite.9,17,34–38

The delayed kinetics of expression that we observed in mRNA accumulation and protein production are consistent with the concept that NOS2 is not directly upregulated by IFN-γ but rather by an IFN-γ inducible transcription factor, Interferon Response Factor-1 (IRF-1).59 The promoter region of NOS2 in other animals and humans is known to contain 20 or more putative cytokine/transcription factor binding sites, including Interferon Response Elements (IREs) for binding IRF-1. It is unclear how many species possess IFN-γ responsive IFN-γ activation sequence (GAS) elements in their NOS2 promoters, thus permitting direct upregulation of transcription by IFN-γ as might be predicted in rats.55

The NOS induction cascade is thought to include several steps. First, IFN-γ binds to its receptor, activating the Jak-Stat signal transduction cascade, resulting in the production of IRF-1 in addition to many other IFN-γ-inducible proteins that contain a GAS element in the regulatory regions of their genes. It has been proposed that IRF-1 then directly interacts with NF-kB, which has been induced by TNF-α.66 The IRF-1/NF-kB direct interaction is believed to result in bending of the DNA in the region of the promoters to which they bind, forming an enhancerosome and thus permitting enhanced transcription.66 This mechanism proposed by Saura et al. would seem to be a reasonable way to explain the synergistic upregulation of NOS by numerous growth factors and transcription factors with binding sites spread over a large promoter/enhancer region.

The downregulation of nitrite production by TGF-β1 and β2 provides the cornea with a means to reduce the destructive potential of NOS2. TGF-β, when given intravitreally, decreases the severity of endotoxin-induced uveitis, a NOS-mediated disease; thus, in rabbits it appears that TGF-β inhibits inducible NOS in vivo, as well.40 TGF-β has been found to downregulate NOS2 and NO production in many cell types, except for bovine corneal stromal and endothelial cells, as well as bovine retinal pigment epithelial cells.12,15,30–33 It is unclear why NO activity in bovine cells responds differently than many other cell types.

TGF-β has been shown to affect nitrite production by inhibiting transcription, by destabilizing mRNA, by altering enzyme activity/stability, and by undefined posttranscriptional mechanisms.53 The mechanism of inhibition appears to vary with cell type and species. Our data clearly document a dose-dependent inhibition of nitrite accumulation by TGF-β2 in rabbit corneal stromal cells. Our data (not shown) also demonstrate inhibition of nitrite accumulation by TGF-β1. The steady state levels of transcripts appeared similar in stromal cells treated with varying doses of TGF-β1 or β2, thus the mechanism of inhibition appears to be at the posttranscriptional level. Western blot analyses of IFN-γ document reduced steady state levels of protein, thus indicating inhibition of translation or reduced protein stability. Cultured rabbit and human corneal stromal cells express TGF-β receptors that appear functional.11,42 Although TGF-β is known to induce the differentiation of stromal fibroblasts to myofibroblasts, cells in our cultures appear to remain as fibroblasts, expressing αSMA, integrin and low amounts of α smooth muscle actin, which does not organize into highly visible stress fibers. Our cells would be considered cultured under high to intermediate density as described by Petrividou et al., whose work suggests that the TGF-β signal transduction cascade would be functional in our cells.

Few published studies have addressed the function of nitric oxide synthase in corneal cells or its role in corneal diseases. The first indication that an isoform of NOS was present in corneas was from studies demonstrating diaphorase activity in the corneal epithelium and endothelium, but not stroma, of normal corneas. Diaphorase staining is a measure of NADPH oxidase activity in partially fixed tissues. Diaphorase activity in some cases is due to NOS activity, and in other cases it is due to other enzymes. Numerous controls of diaphorase-stained tissue are required to directly relate the activity to NOS. Based on the evidence provided, only a small portion of the diaphorase activity observed by Osborn et al. could be attributed to NOS. We have observed NADPH diaphorase activity not only in the corneal epithelium and endothelium but also in the stromal cells of normal corneas; however, we could not consistently inhibit the activity with NOS-specific inhibitors (data not shown). Great care should be exercised in associating NADPH diaphorase activity with NOS in the cornea. We chose to develop specific probes to detect NOS expression in cultured cells and to use them in combination with monoclonal antibodies, isoform-specific inhibitors, and nitrite production to examine NOS production. Our data clearly document NOS2 induction and demonstrate its response to cytokines in cultured corneal stromal cells. Corneal endothelial cells in culture upregulate NO production only slightly and do not contain NOS1 or NOS3 at detectable levels in our hands. Corneal epithelial cells produced little evidence of NOS in any form. The literature documents at least three potential roles for NO and NOS2 in the cornea, including (a) inhibition of neovascularization, (b) regulation of corneal hydration, and (c) mediation of tissue damage.

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References


