# Doxycycline Inhibition of Interleukin-1 in the Corneal Epithelium

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**Purpose.** To evaluate the effect of doxycycline on the regulation of interleukin (IL)-1 expression and activity in human cultured corneal epithelium.

**Methods.** Human corneal limbal epithelium (HLE) was cultured from explants prepared from limbal rings of donor corneas. Primary cultured limbal epithelial cells were treated with either 10 $\mu$g/ml lipopolysaccharide (LPS), LPS with 10 $\mu$g/ml doxycycline, or LPS with 0.1 mg/ml methylprednisolone (MP) for 24 hours. The intracellular and supernatant protein amounts of IL-1$\alpha$, the precursor and mature forms of IL-1$\beta$, IL-1 receptor antagonist (IL-1 RA), and the intracellular level of IL-1$\beta$–converting enzyme (ICE) were measured with enzyme-linked immunosorbent assays (ELISAs). Western blot analysis was performed to evaluate IL-1 RA protein. mRNA steady state amounts were determined by RNase protection assay (RPA) for IL-1$\alpha$, IL-1$\beta$, IL-1 RA, and ICE.

**Results.** LPS increased the mRNA and protein amounts of intracellular and released IL-1$\alpha$, mature IL-1$\beta$, and IL-1 RA. Doxycycline inhibited the LPS-induced IL-1$\beta$ increase in the mRNA and protein amounts in the corneal epithelium and upregulated the expression of the anti-inflammatory IL-1 RA protein. In addition, doxycycline reduced the steady state level of the cellular ICE protein but did not affect the level of ICE transcripts. IL-1$\beta$ secreted to the conditioned media of HLE was functionally active in inducing matrix metalloproteinase (MMP)-1 and MMP-3 in cultured corneal fibroblasts. Doxycycline significantly decreased IL-1$\beta$ bioactivity in the supernatants from LPS-treated corneal epithelial cultures. These effects were comparable to those induced by the corticosteroid, MP.

**Conclusions.** Doxycycline can suppress the steady state amounts of mRNA and protein of IL-$\beta$ and decrease the bioactivity of this major inflammatory cytokine. These data may partially explain the clinically observed anti-inflammatory properties of doxycycline. The observation that doxycycline was equally potent as a corticosteroid, combined with the relative absence of adverse effects, makes it a potent drug for a wide spectrum of ocular surface inflammatory diseases. (*Invest Ophthalmol Vis Sci.* 2000;41:2544–2557)

Keratitis sicca, the corneal epithelial disease that develops in dry eye, is among the most common and problematic conditions faced by ophthalmologists. In mild cases, it is associated with symptoms of irritation, redness, and blurred vision. In the more severe forms, sight-threatening corneal problems may develop, such as filamentary keratitis, corneal epithelial erosions, corneal stromal vascularization, and ulceration. The exact mechanism by which keratitis sicca develops has not been established. Our group has reported that inflammation may be the primary factor causing this condition.[1] The proinflammatory cytokine interleukin (IL)-1 has been identified as a factor that may play a key role in the initiation and perpetuation of this inflammation. We have observed that as tear clearance from the ocular surface decreases, the concentrations of both isoforms of the proinflammatory cytokine IL-1, IL-1$\alpha$,[2] and IL-1$\beta$ (Solomon et al., unpublished results, 2000), increase in the tear fluid. The IL-1 gene family is a group of potent cytokines that function as major mediators of inflammation and immune response.[3] This family is composed of three forms: two proinflammatory forms, IL-1$\alpha$ and IL-1$\beta$, each having a precursor form, and an anti-inflammatory form, IL-1 receptor antagonist (IL-1 RA).

Recent data suggest that the IL-1 cytokines play an important role in the regulation of inflammation and wound healing on the ocular surface. IL-1$\beta$ was found in the epithelium, stroma, and endothelium of the cornea, at the mRNA and protein levels.[4] Type 1 receptor for IL-1 is expressed in stromal fibroblasts.[5] Both IL-1$\alpha$ and IL-1$\beta$ have been found to modulate matrix metalloproteinase (MMP) expression by corneal stromal fibroblasts[6] and their own synthesis in keratocytes,[7] to regulate apoptosis of keratocytes in response to corneal epithelial wounding,[8] and to upregulate hepatocyte growth factor and keratocyte growth factor in corneal fibroblasts.[4] These findings

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make IL-1 a prime candidate for inducing ocular surface disease, especially the chronic subclinical ocular surface inflammation of dry eye.

Traditional therapies for keratoconjunctivitis have consisted of artificial tears and aqueous-conserving therapies, such as punctal occlusion. Although these therapies transiently improve irritation symptoms, they are often ineffective in treating the severe complications of dry eye, such as recurrent corneal epithelial erosion and corneal stromal ulceration. Therapies targeting the underlying inflammatory environment of the ocular surface would represent a major improvement in the management of these conditions and would have a major clinical impact. Consistent with the concept that inflammation is a key feature in the pathophysiology of keratitis sicca is the finding that both aqueous tear deficiency and meibomian gland disease are effectively treated with the corticosteroid methylprednisolone (MP).[9,10] Unfortunately the long-term use of topical corticosteroids is limited by potential sight-threatening side effects, such as glaucoma and cataract. Therefore, there is a clinical need for nontoxic steroid-sparing anti-inflammatory therapies that target IL-1 expression in the corneal epithelium.

Systemically administered tetracycline antibiotics have long been recognized as effective therapies for ocular surface inflammatory diseases. The semisynthetic tetracycline, doxycycline, has been reported to successfully treat the common dry eye condition acne rosacea,[11] as well as recurrent corneal erosions[12] and phlyctenular keratoconjunctivitis.[13] We hypothesized that one of the mechanisms of action of doxycycline in dry eye is the downregulation of the IL-1–mediated inflammatory cascade in the corneal epithelium. Therefore, the purpose of this study was to evaluate the effect of doxycycline on the regulation of IL-1 expression and activity in the human corneal epithelium.

## METHODS

### Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), HEPES buffer, F12 (Ham's), were from Life Technologies (Rockville, MD). Tissue culture plates were from Becton Dickinson (Franklin Lakes, NJ). Cholera toxin subunit A, epidermal growth factor (EGF), hydrocortisone, LPS (derived from Serratia marcescens), doxycycline, and MP were from Sigma (St. Louis, MO). IL-β precursor, IL-1β–converting enzyme (ICE), and IL-β mature enzyme-linked immunosorbent assay (ELISA) kits were from Cistron (Pine Brook, NJ); IL-1 α and IL-1 RA ELISA kits were from R&D systems (Minneapolis, MN); and MMP-1 and MMP-3 ELISA kits were from Oncogene Research Products of Calbiochem (Cambridge, MA). RNA lysis and RNase protection kits were from Ambion (Austin, TX). IL-1 RA was from Genzyme (Cambridge, MA). The BCA protein assay kit was from Pierce (Rockford, IL).

### Culture of Human Corneal Limbal Epithelium

Human corneal limbal epithelium (HLE) was cultured from explants of human donor corneoscleral rims, provided by the Florida Lions Eye Bank at the Bascom Palmer Eye Institute. Each corneoscleral rim was trimmed, the endothelial layer and iris remnants were removed, and the tissue was treated with dispase for 15 minutes. Each rim was dissected into 12 equal parts, which were applied to six-well plastic dishes and covered with a drop of FBS overnight. The explants were cultured in supplemented hormonal epithelial medium (SHEM) containing equal amounts of DMEM and Ham's F12 medium, supplemented with 5% FBS, 0.5% dimethyl sulfoxide, 2 ng/ml EGF, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 0.5 μg/ml hydrocortisone, 30 ng/ml cholera toxin A, 50 μg/ml gentamicin, and 1.25 μg/ml amphotericin B. Cultures were incubated at 37°C under 95% humidity and 5% $CO_2$. The medium was changed every 2 days. Cultures were maintained for 10 to 14 days until confluence and then switched to the serum-free medium described above, without FBS, for 24 hours before the additions of treatments.

To demonstrate the effect of doxycycline on the corneal epithelium and to compare it with that of a corticosteroid, primary cultures of HLE were treated with 10 μg/ml bacterial LPS alone or in combination with either 0.1 mg/ml MP or 10 μg/ml doxycycline. These treatments were maintained for 24 hours.

After a 24-hour treatment, the culture supernatant was collected from each well, centrifuged, and stored in −80°C until assayed by ELISA. Cell lysis solution, containing 50 mM Tris-HCl (pH 7.6), 300 mM NaCl, and 0.5% Triton X-100, was added to the cells for 3 hours, and the cellular protein was collected, centrifuged, and stored in −80°C until assayed by ELISA. In parallel cultures, the cells were subjected to lysis buffer (Direct Protect; Ambion), and total RNA was isolated for further assessment by RNase protection assay (RPA). The ELISA and RPA were targeted at determining the protein and mRNA levels, respectively, of IL-1α, IL-1β, IL-1 RA, and ICE.

### RPA Template Construction

Partial cDNAs for human IL-1α, IL-1β, IL-1 RA, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were prepared by reverse transcription–polymerase chain reaction (RT-PCR). PolyA+ RNA was isolated from cultured human corneal epithelial cells using oligo-dT–coated beads (Oligotex Direct mRNA Isolation System; Qiagen, Valencia, CA), according to the manufacturer's instructions. RT was performed using 200 ng mRNA as template and gene-specific primers (see Table 1) were prepared to human IL-1α (gene accession, X02531), IL-1β (gene accession, X02532), IL-1 RA (gene accession, M63099), and GAPDH (gene accession, NM 002046), according to the manufacturer's instructions (Superscript II Reverse Transcription Kit; Life Technologies). The resultant first-strand cDNA was used for PCR (PCR Kit, Life Technologies) using a gene-specific upstream primer and the same downstream primer-2 used for RT. An aliquot of the initial PCR reaction (except for the GAPDH probe that required only a single round of PCR) was reamplified using the same upstream primer and a third gene-specific primer, downstream primer-1. The primers used in this second amplification were designed with 12 nucleotide additions (four copies of a trinucleotide repeat containing a single deoxyuracil in each repeat) at their 5′ ends, to facilitate rapid cloning of the amplimers (pAMP1 System; Life Technologies).

### RPA Probes

RPA requires hybridization of sense mRNA to complementary radiolabeled antisense RNA. Subsequently, double-stranded

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**Table 1.** Primers Used in RNAse Protection Assay

| cDNA | Size (bp) | Upstream Primer (5′–3′) | Downstream Primer-1 (5′–3′) | Downstream Primer-2 (5′–3′) |
|---|---|---|---|---|
| IL-1$\alpha$ | 407 | CAA GGA GAG CAT GGT GGT AGT AGC AAC CAA CG | GCA CTG GTT GGT CTT CAT CTT GGG C | TAG TGC CGT GAG TTT CCC AGA AGA AGA GGA GG |
| IL-1$\beta$ | 348 | GCT ACG AAT CTC CGA CCA CCA CTA CAG C | CCT TGT ACA AAG GAC ATG GAG AAC ACC | CTT ATC ATC TTT CAA CAC GCA GGA CAG G |
| IL-1 RA | 308 | CCA TTC AGA GAC GAT CTG CCG ACC | GCT TGT CCT GCT TTC TGT TCT CGC | CTG TCT GAG CGG ATG AAG GCG AAG C |
| GAPDH | 188 | GAC ATC AAG AAG GTG GTG AAG CAG GC | | CCA AAT TCG TTG TCA TAC CAG GAA ATG AGC |

mRNA-radiolabeled antisense RNA hybrids are treated with RNase specific for single-stranded RNA. Protected hybrids can then be resolved and quantified using gel electrophoresis and autoradiography. Radiolabeled antisense RNA was transcribed using the a kit (Maxiscript T7; Ambion) and labeling with [$\alpha$-$^{32}$P]CTP (800 Ci/mmol). Plasmids were digested at a unique *Bam*HI site upstream of the cloned cDNAs. RNA probes were generated for IL-1$\alpha$, IL-1$\beta$, and IL-1 RA, and GAPDH. The GAPDH message is a housekeeping gene that is found to be expressed 10 to 20 times more than the messages for the cytokines measured in the RPA. The GAPDH probe was therefore transcribed to yield a specific activity 10 times less than that of the cytokine probes, to allow simultaneous detection of protected cytokine probe fragments as well as GAPDH probe fragments, given the range of sensitivity provided by the x-ray film. After transcription, probes were DNase treated to remove template DNA, and unincorporated nucleotides were removed using RNA Quick Spin columns (Roche Molecular Biochemicals, Indianapolis, IN). A template set containing DNA templates for ICE and GAPDH RNA probes, was purchased from PharMingen (San Diego, CA).

### RNase Protection Assay for IL-1$\alpha$, IL-1$\beta$, IL-1 RA, and ICE

RNase protection assays were performed (Direct Protect System; Ambion) as described by the manufacturer. Briefly, cultured human corneal epithelial cells were resuspended in lysis buffer at approximately $10^7$ cells/ml. The cell lysis buffer included concentrated guanidine thiocyanate, which rapidly solubilizes cells and also rapidly inactivates ribonucleases. Assays were performed using 50 $\mu$l of cell lysate, $10^5$ cpm of each cytokine probe (specific activity, $5 \times 10^{5}$ cpm/$\mu$g) and $4 \times 10^4$ cpm of the GAPDH probe (specific activity, $5 \times 10^{4}$ cpm/$\mu$g) for each sample. Samples were allowed to hybridize overnight at 37°C. They were then treated for 30 minutes at 37°C with RNase solution, after which the RNase was inactivated with proteinase K. Protected RNA fragments were precipitated and separated on a 6% polyacrylamide urea-Tris-base, boric acid, EDTA (TBE) sequencing gel.

RPAs for IL-1$\alpha$, IL-1$\beta$, and IL-1 RA were repeated four times on primary cultures derived from four different donor corneas. RPAs for ICE were repeated three times on primary cultures from three donor corneas. Autoradiographs from these gels were scanned and then analyzed using image analysis software (Gel-Pro; Media Cybernetics, Silver Spring, MD). The digitized data for each band was plotted, and the area under the curve for each peak was calculated with statistical software (GraphPad Prism; GraphPad Software, San Diego, CA). The value for each cytokine band was divided by the corresponding value of the GAPDH band in the same lane to calculate the relative mRNA amount for each gene. Results are shown as means ± SEM of relative mRNA amounts from three or four experiments.

### Immunodetection of IL-1$\beta$ Precursor, IL-1$\beta$ Mature, IL-1$\alpha$, IL-1 RA, and ICE

The conditioned media and cell lysates of corneal limbal epithelial cells from four independent primary cultures, derived from four different donor corneas were collected, centrifuged, and stored at −80°C until assayed. The concentrations of IL-1$\beta$ precursor and IL-1$\beta$ mature in the cell lysates and in the supernatants and of ICE in cell lysates were measured by ELISAs according to the respective manufacturer's protocol. The cellular protein concentration in cell lysates was measured with the BCA protein assay kit.

### Western Blot Analysis for IL-1 RA

To evaluate the expression of IL-1 RA protein in the conditioned medium and in the cells, we further incubated primary limbal epithelium with LPS, LPS and MP, LPS and doxycycline, or LPS with MP and doxycycline, using the same concentrations as described earlier.

Cell lysates and conditioned media containing equal quantities of protein were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using 4% to 15%, 0.75-mm thick polyacrylamide gel (Mini-ready; Bio-Rad, Hercules, CA) at a constant 200 V for 45 minutes, in a mini-protean electrophoresis apparatus (Bio-Rad). A positive control (human recombinant IL-1 RA; R&D) and prestained (7.5–203 kDa) molecular weight protein markers (Bio-Rad) were run simultaneously with the samples. Resolved proteins were transferred to nitrocellulose membranes (BioTrance NT, Ann Arbor, MI) using a minitank blot apparatus (Bio-Rad). Membranes were blocked in 3% fat-free milk for 45 minutes After a 1-hour incubation with polyclonal rabbit anti-human IL-1 RA antibody diluted in 1% bovine serum albumin, Tris-buffered saline, and 0.5% Tween 20, the membranes were incubated with IgG-horseradish peroxidase–conjugated goat anti-rabbit (Sigma) diluted 1:80,000 in 1% bovine serum albumin, Tris-buffered saline, and 0.5% Tween 20. Signals were detected with an immunodetection kit (Renaissance Enhanced Chemiluminescence [ECL]; NEN Life Science Products, Boston, MA), and

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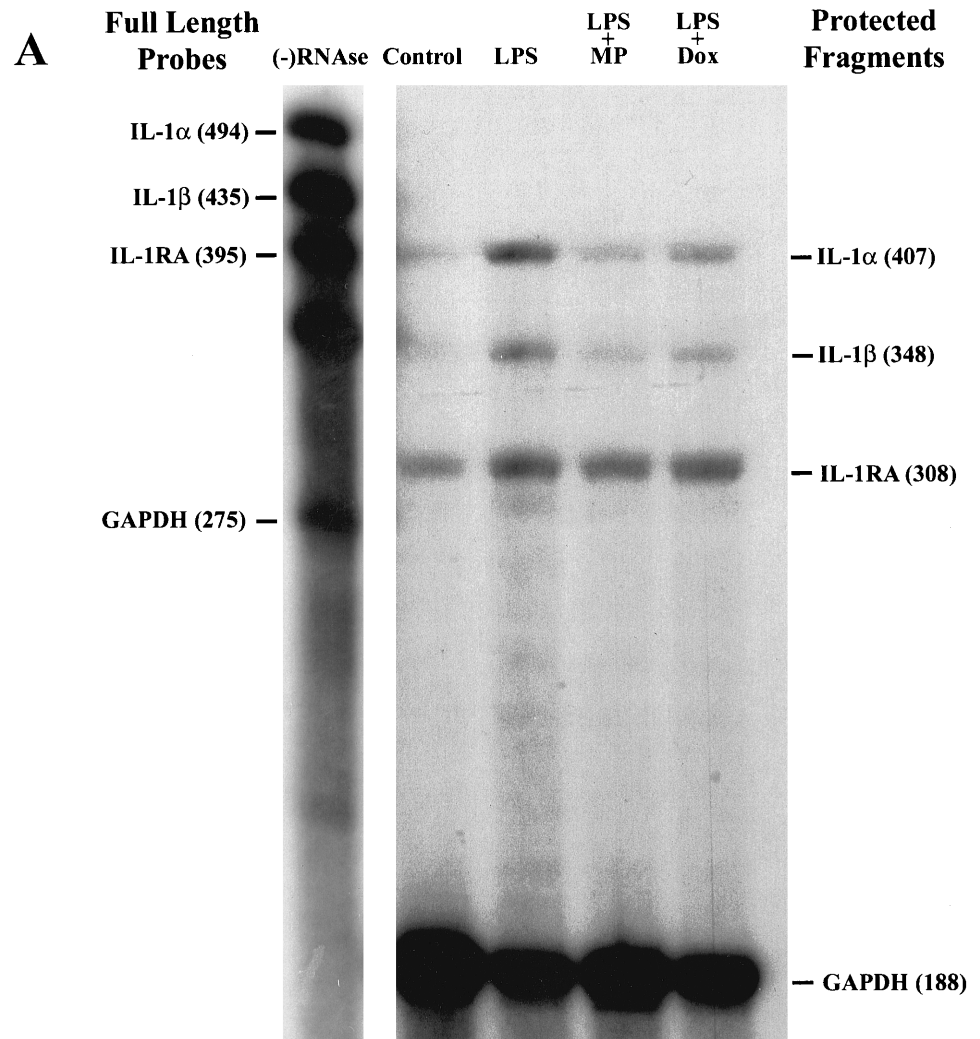


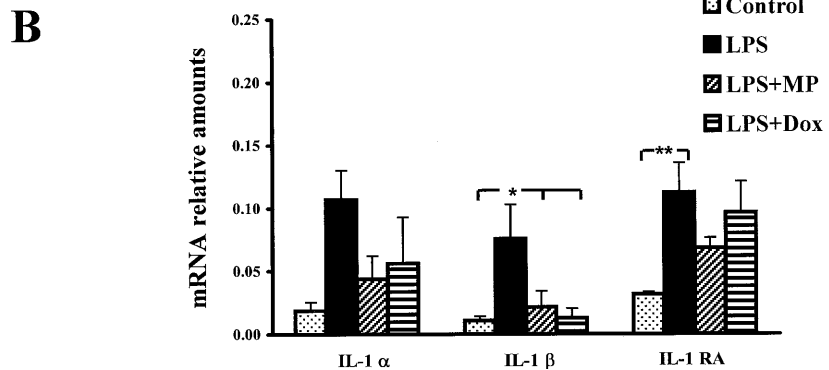


**FIGURE 1.** (**A**) RNase protection assay of RNA extracted from primary cultured human corneal epithelial cells. Cells were treated with either 10 $\mu$g/ml bacterial LPS alone or in combination with either 0.1 mg/ml MP (LPS + MP) or 10 $\mu$g/ml doxycycline (LPS + Doxy). (**B**) mRNA amounts for IL-1$\alpha$, IL-1$\beta$, and IL-1 RA corrected for the different amounts of GAPDH. Data are the mean ± SEM of four different experiments on primary cultures from four different donor corneas. A significant increase in the mRNA amounts of IL-1$\beta$ was observed after treatment with LPS, with a subsequent significant decrease to the control level when either MP or doxycycline was added to LPS (*$P = 0.037$, ANOVA). Similar nonsignificant changes occurred in the IL-1$\alpha$ mRNA expression. The mRNA amounts of IL-1 RA were significantly increased by LPS (**$P = 0.017$, ANOVA) but were not markedly changed with the addition of MP or doxycycline.

then exposed to x-ray film (Eastman Kodak, Rochester, NY) from 30 seconds to 3 minutes.

### IL-1$\beta$ Activity Assay

To evaluate the functional activity of IL-1$\beta$ secreted by cultured HLE, we sought to develop a bioassay that is based on the induction of MMP-1 and MMP-3 in corneal fibroblasts by IL-1$\beta$.[6] IL-1$\beta$ has been previously demonstrated to induce MMP-1 and MMP-3 secretion in human synovial fibroblasts,[14] endometrial stromal cells,[15] and fibrochondrocytes.[16] We therefore cultured early-passaged corneal fibroblasts in conditioned media that were collected from the HLE cultures. The resultant MMP-1 and -3 supernatant concentrations were measured.

Corneal fibroblasts were cultured as previously described.[17] Briefly, the central corneas of donor eye bank eyes were isolated with a 6-mm trephine after removal of the epi-

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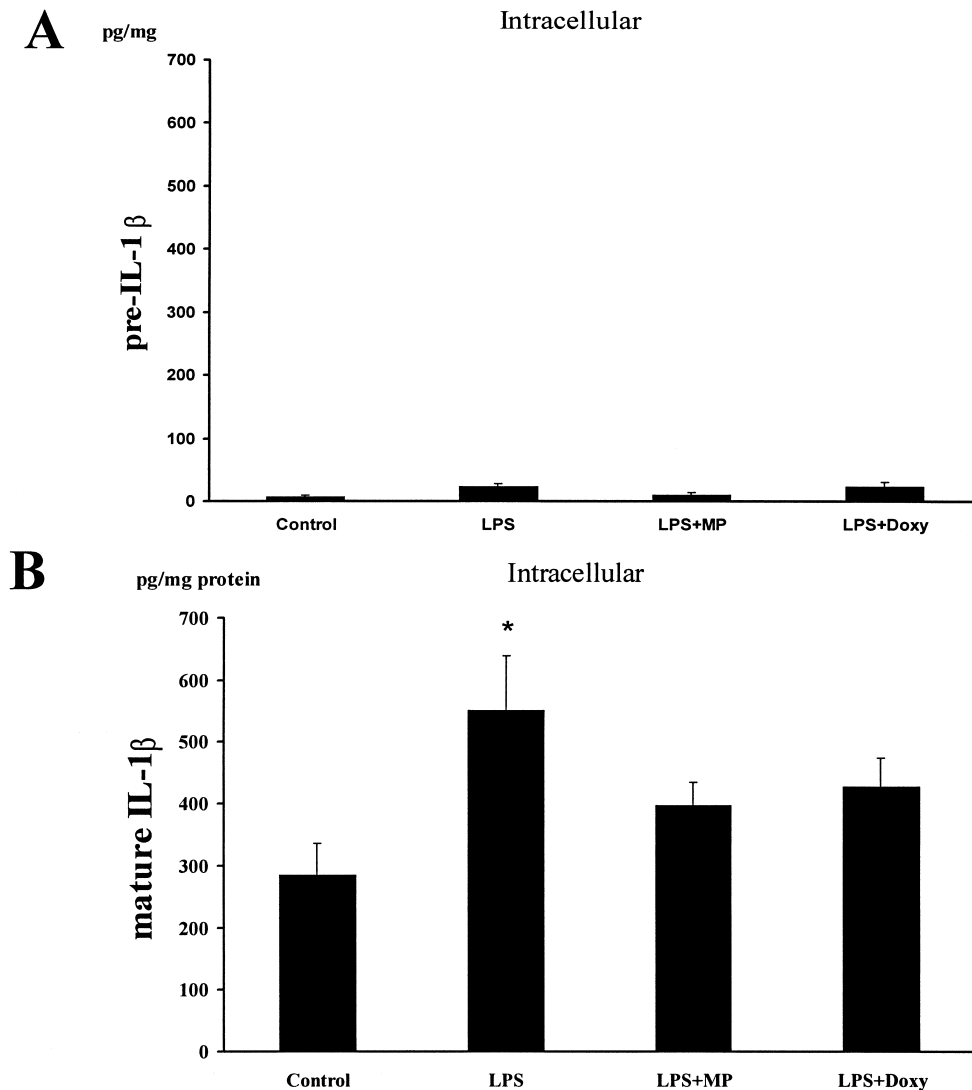


**FIGURE 2.** Cellular protein amounts of the precursor (**A**) and the mature (**B**) forms of IL-1$\beta$ measured by ELISA in cell lysates of HLE collected after 24 hours in serum-free medium and stimulated with either bacterial LPS, LPS + MP, or LPS + doxycycline (LPS + Doxy). Protein amounts are expressed in picograms per milligram of total cellular protein assayed in corresponding cellular lysates. Data are the mean ± SEM from four independent experiments performed in HLE cultures from four different donor corneas. LPS induced a significant increase in the intracellular concentration of the mature IL-1$\beta$ protein, with subsequent decrease when MP or doxycycline was added (*$P = 0.035$, ANOVA).

thelium and the endothelium with a cell scraper. Explant cultures were prepared in the same manner as described earlier for limbal epithelial culture, except that DMEM containing 10% FBS (D-FBS) was used. Cultures were incubated at 37°C under 95% humidity and 5% $CO_2$, and the medium was changed twice a week. Fibroblasts were subcultured with 0.05% trypsin and 0.85 mM EDTA in a calcium-free MEM at 80% to 90% confluence with 1:3 to 1:4 split for three passages.

Third-passage fibroblasts were seeded in six-well tissue culture plates at a density of $2 \times 10^5$ cells per well. After 5 days in culture, on confluence, cultures were switched to a serum-free medium containing DMEM supplemented with 5 $\mu$g/ml insulin, 5 $\mu$g/ml transferrin, 5 ng/ml selenium, 50 $\mu$g/ml gentamicin, and 1.25 $\mu$g/ml amphotericin B (D-ITS). Some cultures were maintained in D-FBS. After a 24-hour incubation in D-ITS, cultures were divided into two groups.

The first group of cultures served as a positive control and were treated directly with human recombinant IL-1$\beta$ with one of the following: D-ITS alone, recombinant human (rh)-mature IL-1$\beta$ (10 ng/ml), rh-pre-IL-1$\beta$ (10 ng/ml), and rh-pre-IL-1$\beta$ (10 ng/ml) with matrix MMP-9 (1 $\mu$g/ml).

The second group of cultures were treated with conditioned media (CM) derived from HLE cultures that had been treated as described earlier. These treatments included CM from HLE culture treated with medium alone (CM-SHEM), CM from HLE culture treated with LPS (CM-LPS), CM from HLE culture treated with LPS and doxycycline (CM-LPS + doxy), and CM from HLE culture treated with doxycycline (CM-doxy). To exclude the possibility that the drugs contained in the conditioned media (LPS and doxycycline) altered MMP secretion in corneal fibroblasts, two additional treatments were added: SHEM with LPS (10 $\mu$g/ml; SHEM-LPS) and SHEM with LPS and doxycycline (10 $\mu$g/ml each; SHEM-LPS + doxy).

Cultures were incubated with one of these treatments for 24 hours, and thereafter their supernatants were collected for measurement of MMP-1 and MMP-3 concentrations by ELISAs.

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