Expression of NADPH Oxidase in Rabbit Corneal Epithelial and Stromal Cells in Culture

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Purpose. Reactive oxygen- and nitrogen-containing molecules produced in high concentrations are mediators of tissue damage caused by inflammation. The free radical molecules superoxide ($O_2^{\cdot-}$) and nitric oxide ($NO^{\cdot}$), when produced at low concentrations, may function as second messengers or regulators of signal transduction. The purpose of these studies was to determine whether corneal epithelial and stromal cells are capable of producing $O_2^{\cdot-}$ via an NADPH oxidase complex.

Methods. Rabbit corneal epithelial and stromal cells, grown as primary cultures and low-passage isolates, were used as the sources of RNA for RT-PCR with primers specific for mRNAs encoding the proteins that comprise an NADPH oxidase complex. The RT-PCR products were sequenced to confirm their identities. The production of proteins composing the oxidase complex was confirmed, and the proteins were identified by Western blot analysis. The production of superoxide in cell-free preparations was assessed by measurement of NADPH-dependent superoxide dismutase (SOD)–inhibitable cytochrome *c* reduction and by electron paramagnetic resonance (EPR) with a superoxide specific spin trap.

Results. Cell-free extracts of corneal epithelial and stromal cells produced superoxide in an NADPH-dependent manner, and this production was inhibited by SOD. EPR confirmed the identity of the reaction product as superoxide anion. Both rabbit corneal epithelial and stromal cells constitutively produced mRNAs encoding five proteins known to comprise a classic neutrophil-like NADPH oxidase complex. Production of NOX4, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ was confirmed by Western blot. Both epithelial and stromal cells expressed isoforms of Rac, a putative regulator of the activity of the complex.

Conclusions. A constitutively expressed NADPH oxidase complex that includes NOX4 is a source of $O_2^{\cdot-}$ produced by rabbit corneal epithelial and stromal cells. Superoxide produced by the oxidation of NADPH via the NADPH oxidase complex is a potential contributor to signal transduction pathways as well as a potential participant in processes that occur during inflammation. (*Invest Ophthalmol Vis Sci.* 2006;47:853–863) DOI:10.1167/iovs.05-1063

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Superoxide anion ($O_2^{\cdot-}$) is one of a group of molecules referred to as reactive oxygen species (ROS). Superoxide by itself is not a strong oxidant, but its reaction products are often extremely reactive. It may react to form molecules such as hypochlorite that are beneficial to infected tissues, or it may react to form molecules such as peroxynitrite and hydrogen peroxide that may be either beneficial or detrimental to cells.[1] Superoxide can be produced in cells by several enzyme systems including xanthine oxidase, uncoupled nitric oxide synthase, complexes I or III of the mitochondrial electron transport system, uncoupled NADPH cytochrome P450 reductase, or NADPH oxidase.[2–5] NADPH oxidase is the protein complex responsible for the large oxidative burst produced by neutrophils. Other types of cells contain slightly modified forms of NADPH oxidase complexes.[6] Complexes containing homologues of oxidase proteins are responsible for the production of $O_2^{\cdot-}$ in cardiac myocytes, dermal epithelial cells, and kidney epithelial cells.[7] Several functions have been proposed for the $O_2^{\cdot-}$ produced by these neutrophil-like NADPH complexes, including roles in apoptosis, senescence, cell proliferation, oxygen sensing, signal transduction, and regulation of hormone synthesis.[6,8]

In the cornea, there are multiple potential sources of $O_2^{\cdot-}$ that may be present, not only during inflammation but also in the healthy tissue. One enzyme capable of producing $O_2^{\cdot-}$ is xanthine oxidase, which is present in rabbit and human epithelial, stromal, and endothelial cells.[2] Superoxide may also be produced by uncoupled NADPH cytochrome P450 reductase, an enzyme known to be present in the corneal epithelium.[9] It is unlikely that uncoupled NOS3 could be a source of $O_2^{\cdot-}$ in corneas that are not neovascularized. We have been unable to document NOS3 expression in corneal cells, but clearly NOS2 expression occurs—also a potential source of $O_2^{\cdot-}$.[10] Complexes I and III of the mitochondrial electron transport chain are a fourth potential source of $O_2^{\cdot-}$ in cells; however, it is unclear to what extent these complexes produce $O_2^{\cdot-}$ when electron transport is not inhibited or uncoupled.[4] The purpose of the work reported herein was to determine whether corneal epithelial and stromal cells are capable of producing $O_2^{\cdot-}$ via an NADPH oxidase complex.

Functional NADPH oxidase complexes are composed of up to six proteins. In neutrophils, NADPH complexes contain two membrane-bound proteins, $gp91^{phox}$ (NOX2) and $p22^{phox}$, which are constitutively expressed. These two proteins comprise a complex known as cytochrome b_{558}.[11] On stimulation, the constitutively expressed cytoplasmic components of the complex, which include $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$, become tethered to the NOX2-$p22^{phox}$ complex.[12,13] The assembled complex is then capable of producing $O_2^{\cdot-}$ by oxidation of NADPH. The small guanosine triphosphate (GTP)–binding protein Rac is essential for activity and may regulate the amount of $O_2^{\cdot-}$ produced.[14,15] Some cells that are not of myeloid origin are capable of producing $O_2^{\cdot-}$ in a constitutive manner by means of an NADPH oxidase complex that appears to be assembled and associated with the triton-insoluble fraction of the cell.[16] The proteins of these homologous complexes, as they exist in nonmyeloid cells may contain any of several

TABLE 1. Primers Used in the Studies of NADPH Oxidase in Rabbit Corneal Epithelial and Stormal Cells

Primer Name	Sequence 5′–3′	Product (bp)	Reference
HuNOXI-S1	GCCTGTGCCCGAGCGTCTGC	522	AJ438989*
HuNOXI-AS2	ACCAATGCCGTGAATCCCTAAGC		
GP91PHOX-S1	GGAGTTTCAAGATGCGTGGAAACTA	549	Cheng et al.[17]
GP91PHOX-AS2	GCCAGACTCAGAGTTGGAGATGCT		
HuNOX3-S1-2	CCATGGGACGGGTCGGATTGT	376	NM_015718*
HuNOX3-AS2-2	GGGGGCAGAGGTAAGGGTGAAGG		
HuNOX4-S1	GTCATAAGTCATCCCTCAGA	797	Shiose et al.[21]
HuNOX4-AS2	TCAGCTGAAAGACTCTTTAT		
NOX5-S1	ATCAAGCGGCCCCCTTTTTTCAC	238	Cheng et al.[17]
NOX5-AS2	CTCATTGTCACACTCCTCGACAGC		
P22PHOX-S1	GTTTGTGTGCCTGCTGGAGT	316	Jones et al.[22]
P22PHOX-AS2	TGGGCGGCTGCTTGATGGT		
P47PHOXR-S1	CTCCCGCTGTCCACACCTGCTGAA	349	AF324409*
P47PHOXR-AS2	GGGCTCTGGGTCCTCTGGCTCGTC		
P67PHOX-S1	TACTTCCAACGAGGGATGCTC	714	Pagano et al.[23]
P67PHOX-AS2	AGCTTTCCTCCTGGGCT		
P67PHOXR-S1	CACGCCCGGATCTGCTTCAAC	363	AF323789*
P67PHOXRAS2	GTGGCGGGGCTCGACTTCAT		
P40PHOXR-S1	TCATCTACCGCCGCTACCGCCAGTTC	473	AF323790*
P40PHOXRAS2	AGTGCCCTCCAGCCAGTCCTTGTTGA		
P40PHOXR2-S1	CCGGAGGAAGATGACCCCACCAACTG	249	AF323790*
P40PHOXR2AS2	GCCTCGCCTGCCTCCACCAT		
MuRAC1-S1	CCTACCCGCAGACAGTTGGAGACAC	395	XM_132485*
MuRAC1-AS2	CTTGACAGGAGGGGGACAGAGAACC		
MuRAC2-S1	GCCCCAGCACCCCCATCATCC	252	NM_009008*
MuRAC2-AS2	AGGGGCGCTTCTGCTGTCGTGTG		
MuRAC3-S1	GCTTCGGCCACTCTCCTATCCTCA	202	AB040819*
MuRAC3-AS2	CTTCTTGTCCCGCAGCCGTTCA		
MamGAPDH-S1	CCATGGAGAAGGCTGGGG	196	Dveksler et al.[24]
MamGAPDHAS2	CAAAGTTGTCATGGATGACC		

* Primers designed in our laboratory using the cDNA sequence indicated.

isoforms of gp91^{phox} (i.e., NOX1 through -5) as well as isoforms of p47^{phox}, p40^{phox}, and p67^{phox} and Rac1, rather than Rac2.[15,17,18] The results of the studies reported herein document that corneal epithelial and stromal cells are capable of producing $O_2^{\cdot-}$ by the oxidation of NADPH via an NADPH oxidase complex. The results show that the complex is composed of NOX4, p22^{phox}, p47^{phox}, p40^{phox}, and p67^{phox} homologue and Rac.

MATERIALS AND METHODS

Cell Culture

Rabbit corneal epithelial and stromal cells were isolated, grown to confluence, and subcultured with modifications to published procedures.[19,20] Primary cultures were established from the corneas of New Zealand White rabbits of either sex. Rabbits were obtained from a local supplier and their corneas determined to be free of defects by slit lamp examination. The animals were housed in animal quarters approved by the American Association for Laboratory Animal Science. The animals were 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 (Roche Diagnostics, Mannheim, Germany) digestion of the anterior portions of excised corneas after removal of the endothelium and posterior stroma.[19] After dispase II digestion at 4°C for 16 hours, the epithelium was removed by gentle scraping and digested to a single-cell suspension by treatment with trypsin-EDTA. Trypsin digestion was terminated with type I-S soybean trypsin inhibitor (Sigma-Aldrich, St. Louis, MO) and the cells harvested by centrifugation. Epithelial cells recovered from a single cornea were seeded into flasks (Primaria; BD Biosciences, Lincoln Park, NJ) and grown to confluence at 34°C in defined keratinocyte serum-free medium (Invitrogen-Gibco, Grand Island, NY) containing 5 μg/mL gentamicin. Corneal stromal cells were isolated from excised corneas, after removal of the epithelium and endothelium by scraping.[20] The stroma was digested for 16 hours at 37°C with 150 U/mL collagenase (*Clostridium histolyticium*; Invitrogen-Life Technologies) in Hanks' balanced salts solution containing penicillin G (100 U/mL) and streptomycin sulfate (100 μg/mL). The cells were recovered by centrifugation, suspended in growth medium, and grown to confluence at 34°C in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose, 5% heat-inactivated defined fetal bovine serum (FBS; HyClone, Logan, UT), 0.1% serum extender (Mito+; Collaborative Biomedical Products, Bedford, MA), and 10 μg/mL ciprofloxacin (Bayer Corp., Kankakee, IL). The cells were passaged at a 1:2 split ratio with 0.05% trypsin and 0.53 mM EDTA and were used in the first four passages.

Reverse Transcription–Polymerase Chain Reaction

RNA was isolated from rabbit epithelial and stromal cell cultures directly by lysis of PBS-rinsed cultures in RLT buffer containing 1% β-mercaptoethanol (Qiagen, Valencia, CA). The cells were dislodged from the plate and each sample (approximately 1×10^6 cells) was homogenized with a spin column (QIAShredder; Qiagen) after the DNA was sheared by passing the lysate through a 18-gauge needle 16 times. Total RNA was then isolated from the cell lysates with mini spin columns (RNeasy; Qiagen). RNA samples were treated with DNase I (GE Healthcare, Piscataway, NJ) at a concentration of 1 U/μg RNA. The samples were digested for 30 minutes at 37°C, followed by heat inactivation of the DNase at 75°C for 10 minutes. cDNA was amplified with sequence-specific primers (Table 1). In some cases, primers were prepared as described by others, and in other cases, primers were designed in our laboratory. Each primer was analyzed by sequence alignments (DNAStar, Inc., Madison, WI), and the products of the

RT-PCR reactions were analyzed by sequencing to confirm that the products were specific. cDNA was generated from 1 to 3 μg total RNA, 5 mM $MgCl_2$, 1 mM dNTPs, 1 U/μL RNase inhibitor (Applied Biosystems, Inc. [ABI], Foster City, CA), 4 U/μL MuLV reverse transcriptase (Ambion, Austin, TX), and 2.5 μM random hexamers and the cDNA was amplified (Ampli*Taq* Gold; ABI). Approximately 4% to 10% of the RT reaction mixture was used in each PCR reaction, containing 1 to 3 mM $MgCl_2$, 200 μM each dNTP, 0.2 to 0.4 μM primers, and 0.05 U/μL polymerase (Ampli*Taq* Gold; ABI) in reaction buffer (10 mM Tris-HCl [pH 8.3] and 50 mM KCl). The PCR was performed at 95°C for 10 minutes and then 40 cycles of 95°C for 1 minute, 54 °C to 62°C for 1 minute, and 72°C for 1 minute (model 480; Perkin-Elmer, Boston, MA). Control reactions were run in the absence of the reverse transcriptase to detect the presence of amplifiable DNA that might be contaminating the RNA preparation. The reaction products were detected on ethidium bromide-stained 2% agarose gels (NuSieve; BioWhittaker, Walkersville, MD). Reaction products were identified by direct sequencing of the products excised from the gels.

Sequencing

PCR products were prepared for cycle sequencing via gel extraction and purification with a gel extraction kit (QIAquick; Qiagen). Cycle sequencing of the purified DNA was performed with dye termination sequencing (Prism Big Dye Terminator v3.1 Cycle Sequencing Kit; ABI). The sequencing reactions were processed on a genetic analyzer (Prism 3100-Avant; ABI). The sequence analysis was performed on computer (DNA Sequencing Analysis Software, ver. 5.1 for Windows XP and 2000 Platforms; Qiagen). Sequence alignments were then performed (SeqMan II sequence analysis software, ver. 5.06; DNAStar, Inc.).

Western Blot Analysis

Cells were removed from flasks in 15 mM HEPES buffer (pH 7.5), containing 145 mM NaCl, 0.1 mM $MgCl_2$, 10 mM EGTA, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 1 mM phenylmethylsulfonylfluoride (PMSF), and 2 mM sodium orthovanadate (Na_3VO_4). Lysates were prepared by freeze-thawing, homogenization (Dounce; Bellco Glass Co., Vineland, NJ), and sonication for two 15-second intervals at 100 W. Cell debris was cleared from the lysates by centrifugation at 200*g* for 15 minutes at 4°C. Lysate fractions were separated into soluble and particulate fractions by centrifugation at 29,000*g* for 30 minutes at 4°C. In some experiments, cells were fractionated by low-speed centrifugation at 1,000*g* and high-speed centrifugation at 100,000*g* for 15 minutes at 4°C. The pellets were resuspended in modified RIPA buffer (50 mM Tris-HCl [7.4 pH], 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM sodium chloride, 1 mM EDTA, 1 mM PMSF, 5 μg/mL aprotinin, 5 μg/mL leupeptin, 1 μg/mL pepstatin, 1 mM Na_3VO_4, and 1 mM sodium fluoride). Protein concentrations were determined with the bicinchoninic [BCA] protein assay reagent kit (Pierce Biotechnology, Rockford, IL). Both the supernatant and particulate fractions were stored at −80°C.

SDS-PAGE was performed on Bis-Tris 4% to 12% gels (NuPAGE; Novex, San Diego, CA) with MES (2-(*N*-morpholino)ethanesulfonic acid) running buffer (Invitrogen, Carlsbad, CA). Proteins were detected on the immunoblots by chemiluminescence (SuperSignal Femto West Chemiluminescent Substrate Kit; Pierce) according to the manufacturer's directions. Blots were developed on autoradiograph film (CL-XPosure; Pierce, GBX developer and fixer solutions; Eastman Kodak, Rochester, NY). Blots were reused after the antibodies and substrates were removed with the Western blot stripping buffer (Restore; Pierce) according to the manufacturer's directions.

Antibodies used in these studies included: $gp91^{phox}$ (no. 611415) and $p67^{phox}$ (no. 69820; BD Biosciences, Palo Alto, CA); NOX4 (SC-21860); MOX1/NOX1 (SC-5821), $p22^{phox}$ (SC-117212), $p47^{phox}$ (SC-14015 and SC17845), $p67^{phox}$ (SC-7663), Rac1 (SC-217), and Rac2 (SC-96; Santa Cruz Biotechnology, Santa Cruz CA); $p47^{phox}$ (05-540), $p40^{phox}$ (05-039), and Rac (05-389; Upstate, Lake Placid, NY); and $gp91^{phox}$ (54.1) and $p22^{phox}$ (44.1) (gifts from Al Jesaitis, Montana State University, Bozeman, MT).

Assay of NADPH Oxidase by Superoxide Dismutase Inhibitable Cytochrome *c* Reduction

NADPH oxidase activity was measured in a cell-free system in a manner similar to that used by others.[25] Cells were grown to confluence, rinsed in PBS, and harvested with trypsin and EDTA. Trypsin was inhibited with type I-S soybean trypsin inhibitor (Sigma-Aldrich), and the harvested cells were washed with PBS at 4°C and resuspended in 20 mM MOPS (-(*N*-morpholino)propanesulfonic acid)-KOH buffer (pH 7.4) containing 250 mM sucrose, 0.1 mM EDTA, 2 μM leupeptin, 1 μM aprotinin, and 2 μM pepstatin. The cells were disrupted after 1 freeze-thaw cycle by two 20-second cycles of homogenization (Dounce; Bellco Glass) and two 15-second cycles of sonication at 100 W. Whole cells were removed by centrifugation at 200*g* for 5 minutes at 4°C. The lysate was fractionated by centrifugation at 29,000*g* for 15 minutes at 4°C. The pellet was then resuspended in 50 mM phosphate buffer (pH 7.4) and stored at −80°C. In some experiments, cell lysates were fractionated by low-speed centrifugation at 1,000*g* and high-speed centrifugation at 100,000*g* for 15 minutes at 4°C. Paired assays were conducted by incubation 20 to 60 μg of protein with 10 mM phosphate buffer 130 mM NaCl, 1 mM EGTA, 10 μM FAD, 2 mM NaN_3, 50 μM oxidized cytochrome *c*, and 10 μM GTPγS. One reaction of each pair contained 200 units of superoxide dismutase (SOD). Reactions were initiated by the addition of NADPH to a final concentration of 200 μM. After 1 hour, activity was measured as the SOD-inhibitable increase in absorbance at 550 nm ($E_{mM} = 21$). Assay conditions were established documenting the linearity of superoxide production with time and protein concentrations.

Superoxide ($O_2^{\cdot-}$) Measurements by Electron Paramagnetic Resonance Spin Trapping

BMPO (5-*tert*-butoxycarbonyl 5-methyl-1-pyrroline *N*-oxide) was synthesized as described previously.[26] Cells were grown, harvested, and lysed as described earlier. Cell lysates containing 100 μg of protein were incubated with 50 mM BMPO in 50 mM phosphate buffer containing 100 μM DTPA (diethylenetriamine pentaacetic acid) for 30 minutes at 37°C. Reactions were initiated by the addition of 300 μM NADPH as the substrate. In the parallel experiments, some inhibitors or enzymes were added as indicated. For comparison, NADH was added as the substrate in some reactions. EPR spectra were recorded at room temperature on a spectrometer (EMX; Bruker BioSpin, GmbH, Rheinstetten, Germany) operating at 9.85 GHz. To increase loading sample volume and signal to noise ratio, a liquid sample cell (Aquax; Bruker) was used. Typical spectrometer parameters were scan range, 100 G; field set, 3500 Gauss (G); time constant, 5.12 ms; scan time, 5.12 seconds; modulation amplitude, 1.0 G; modulation frequency, 100 kHz; receiver gain, 6.32×10^5; and microwave power, 10.0 mW. The EPR spectra were simulated with software developed by Dr. Duling from the Laboratory of Molecular Biophysics, NIEHS (Research Triangle Park, NC).[27]

Statistical Analysis

Means were compared by one-way analysis of variance and the significance of differences among means of treatment groups was determined by the Holm-Sidak method (Sigma Stat 3.0; SPSS Inc., Chicago, IL).

Results

The Production of $O_2^{\cdot-}$ by Cell-Free Preparations of Cultured Corneal Epithelial and Stromal Cells

Treatment of frozen sections of rabbit corneas with dihydroethidium produced intense fluorescence that suggested the production of $O_2^{\cdot-}$ by cells in all layers (not shown). The

putative $O_2^{\cdot-}$ could have been produced by several different enzymes or enzyme complexes. To determine whether NADPH oxidase could be a contributor to cellular $O_2^{\cdot-}$ production, cell lysates of rabbit corneal epithelial and stromal cells were fractionated into soluble and particulate fractions according to established methods used to assess NADPH oxidase activity.[28] $O_2^{\cdot-}$ production was assayed by cytochrome *c* reduction assays and the SOD-inhibitable portion quantitated.[25] Particulate fractions prepared from both rabbit corneal epithelial and stromal cell cultures produced $O_2^{\cdot-}$ in a manner that was NADPH-dependent and diphenyleneiodonium (DPI)-inhibitable (Fig. 1). DPI inhibition indicated that a flavoprotein was involved in the reaction. $O_2^{\cdot-}$ production was not inhibited by allopurinol or 1400W; therefore, xanthine oxidase and NOS2 were not the sources of the activity (Figs. 1A, 1B).[25] L-NAME also did not inhibit $O_2^{\cdot-}$ production, indicating that other isoforms of NOS were probably not the source of $O_2^{\cdot-}$. (Fig. 1).[29] NADH was not an effective alternative substrate and rotenone neither inhibited nor enhanced $O_2^{\cdot-}$ production from NADPH, suggesting that the $O_2^{\cdot-}$ did not arise from the mitochondrial electron transport.[4] There are no known absolutely specific inhibitors of the activity of assembled NADPH oxidase complexes. In particulate fractions of corneal cells isolated by centrifugation at 100,000*g*, $O_2^{\cdot-}$ was produced at rates that were not significantly different from those produced by centrifugation at 29,000*g*.

The production of $O_2^{\cdot-}$ was confirmed by electron paramagnetic resonance (EPR) spin trapping using the highly $O_2^{\cdot-}$ specific spin trap 5-*tert*-butoxycarbonyl 5-methyl-1-pyrroline *N*-oxide (BMPO; Fig. 2).[26] The results documented that the fraction of stromal cells that sedimented at 29,000*g* produced $O_2^{\cdot-}$ in an NADPH-dependent manner that was inhibited by SOD and DPI but was not inhibitable by L-NAME, 1400W, or allopurinol (Fig. 2). Identical results were obtained when fractionated extracts of corneal epithelial cells were used as the source of enzyme (not shown). The hyperfine constants and the ratios of peaks document the formation of the BMPO superoxide adduct.[26] The spectra simulation results indicated that recorded spectra were contributed by BMPO-$O_2^{\cdot-}$ and BMPO-OH• adducts. EPR spectra were inhibited by SOD and not by catalase (Fig. 2), indicating that EPR signals were due to $O_2^{\cdot-}$ and not H_2O_2. Some reductants and enzymes can convert the BMPO superoxide adduct to BMPO hydroxyl adduct; thus, EPR spectra of both the BMPO superoxide and BMPO hydroxyl adducts were detected.[26] These data in combination with the assays of cytochrome *c* reduction show that NADPH oxidase is a major contributor to $O_2^{\cdot-}$ production in corneal epithelial and stromal cells.

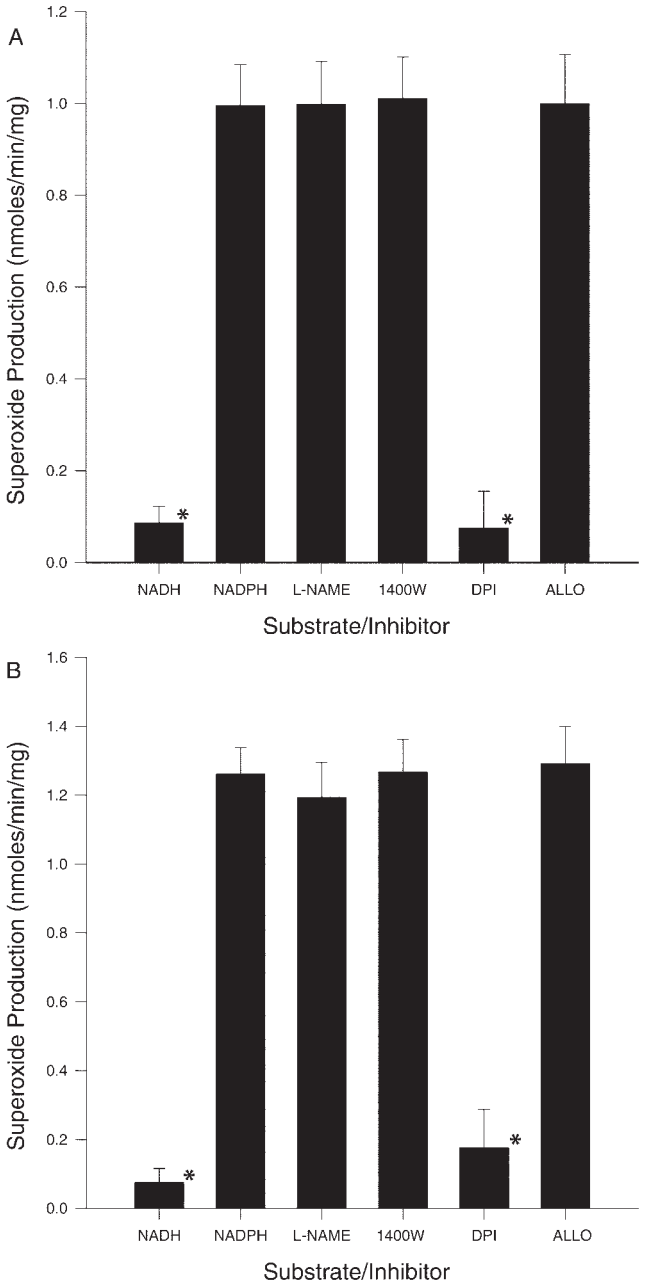

FIGURE 1. Superoxide production as measured by NADPH mediated cytochrome *c* reduction. Rabbit corneal epithelial and stromal cells were grown to confluence, harvested, lysed, and separated into soluble and particulate fractions by centrifugation at 29,000*g*. The pellets were suspended in buffer and used as the sources of the enzyme complex. Superoxide production was assayed by the SOD inhibitable reduction of cytochrome *c*. (**A**) Epithelial cells and (**B**) stromal cells ($n = 3$ each). NADPH was the substrate in assays with the inhibitors L-NAME, 1400W, DPI, and allopurinol. NADH was also used as a substrate. *Significant difference ($P < 0.01$) from the activity as assayed with NADPH as the substrate.

Expression of mRNAs and Proteins of the NADPH Oxidase

RT-PCR primers designed to detect mRNAs of five isoforms of the NOX proteins (i.e., NOX1, -2, -3, -4, and -5) documented that mRNA encoding NOX4 was constitutively present in both rabbit corneal epithelial and stromal cells (Figs. 3A, 4A, respectively). In these cells, there was no detectable mRNA encoding NOX1, -3, and -5. Primers based on the human NOX1 sequence (AJ438989), NOX3 (NM_015718),and NOX5 (AF317889) did not produce detectable products from the RNAs of rabbit corneal epithelial or stromal cells but did produce products from the appropriate positive controls.[17,30]

Two sets of primers were used to detect NOX4. The first set of primers, HuNOX4, were designed based on a human kidney cell sequence NM_016931 that is highly homologous to the NOX4 sequence from human cardiac smooth muscle cells (AF254621).[21,31] NOX 4 sequences of kidney and cardiac smooth muscle cells are 99.8% identical. The HuNOX4 primers amplified a 797-bp product from the cDNA of the 5′ region of the mRNA of NOX4 (Figs. 3A, 4A). Rabbit corneal epithelial and stromal cells produced RNAs that were amplified by RT-PCR with these primers ($n = 6$ each). Neither cell type produced the appropriate 361-bp product from primers derived from the mouse sequence (BC021378). The mouse NOX4 sequence (BC021378) and human NOX4 sequence (AF254621)

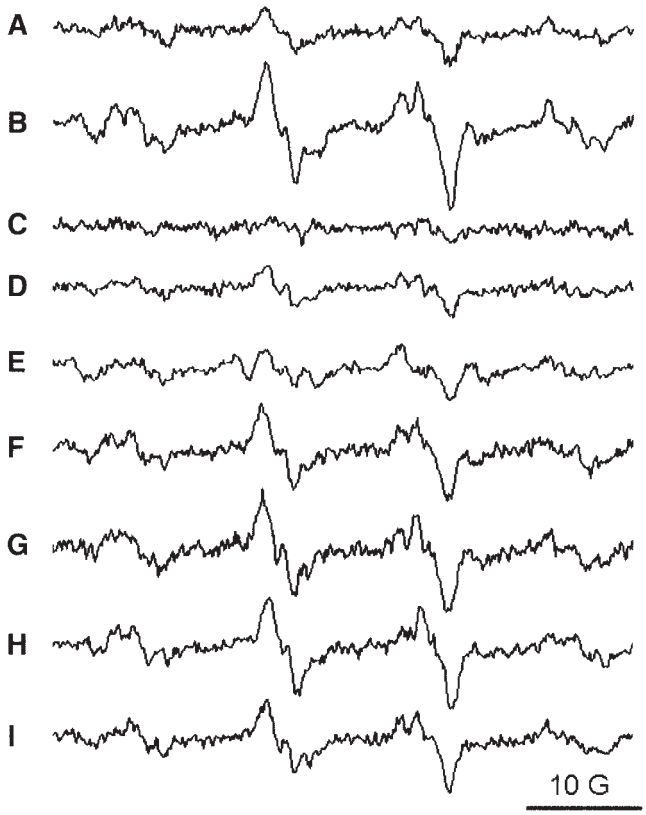

FIGURE 2. EPR spectra of the superoxide adduct of BMPO formed in in vitro assays of NADPH oxidase activity using lysates of rabbit corneal stromal cells as the enzyme source. Cells were grown to confluence, harvested, and lysed, and the lysate was fractionated by centrifugation at 29,000*g*. The pellet was resuspended in 50 mM phosphate buffer with 100 μM DTPA, and the activity of NADPH oxidase was assayed with NADPH (300 μM) as the substrate in the presence or absence of SOD, catalase, or inhibitors. (**A**) Without substrate; (**B**) NADPH as substrate; (**C**) NADPH+SOD (100 U), (**D**) NADPH+DPI (10 μM), (**E**) NADH (300 μM) as substrate (**F**), NADPH as substrate+1400 W (50 μM), (**G**) NADPH+L-NAME (100 μM), (**H**) NADPH+allopurinol (100 μM), and (**I**) NADPH+catalase (300 U).

were 82.7% identical. The sequences of the rabbit corneal RT-PCR products were identical among seven isolates sequenced and were 93% identical with the two human sequences NM_016931 (kidney epithelial) and AF254621 (cardiac smooth muscle; Fig. 5). All but two of the differences between the human and rabbit sequences were in wobble positions, and so we believe that the product amplified represents the product of an NOX4 gene.

Western blot analysis using antibody against a human NOX4 peptide support our hypothesis that an NOX4-driven NADPH oxidase complex was responsible for the O_2^- production by rabbit corneal epithelial and stromal cells (Fig. 6). A protein of approximately 66 kDa, the molecular mass of NOX4, was the dominant band present in membrane-containing fractions (29,000*g* pellets) of both epithelial and stromal cells. Particulate fractions isolated by centrifugation at 100,000*g* also contained the NOX4 protein. A protein of the same molecular weight was detected in the NOX4-positive control cells, HEK293 (Fig. 6).[21] Negative controls prepared from extracts of HL60 cells treated with dimethyl sulfoxide (DMSO) to induce differentiation to neutrophils and freshly isolated rabbit neutrophils did not contain detectable NOX4, as would be expected. Thus, based on our ability to detect NOX4 expression by both RT-PCR and Western blot analysis, we believe that the

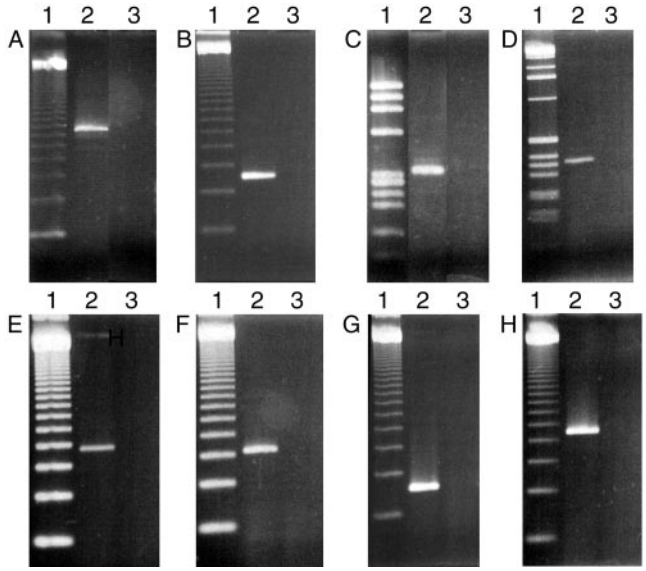

FIGURE 3. Agarose gel electrophoresis of RT-PCR products prepared in reactions RNA from rabbit corneal epithelial cells with primers specific for the genes of the NADPH oxidase complex: (**A**) NOX4, 797 bp; (**B**) $p22^{phox}$, 316 bp; (**C**) $p47^{phox}$, 349 bp; (**D**) $p67^{phox}$, 363 bp; (**E**) $p40^{phox}$, 479 bp; (**F**) Rac1, 395 bp; (**G**) GAPDH, 196 bp; and (**H**) $gp91^{phox}$, 549 bp. *Lane 1*: molecular weight standard; *lane 2*: +RT; *lane 3*: −RT.

NADPH oxidase of rabbit corneal epithelial and stromal cells is NOX4 driven.

Primers specific for a second NOX protein, $gp91^{phox}$, also known as NOX2, amplified products of the appropriate size (549 bp) suggesting the expression of the $gp91^{phox}$ gene product in corneal epithelial but not stromal cells (Fig. 3H; $n = 9$). RT-PCR products amplified from NOX2 messages from epithelial cells were 100% identical with those reported for rabbit leukocytes.[32] Antibodies to NOX2 did not detect $gp91^{phox}$ in either corneal cell type but did detect $gp91^{phox}$ in the positive

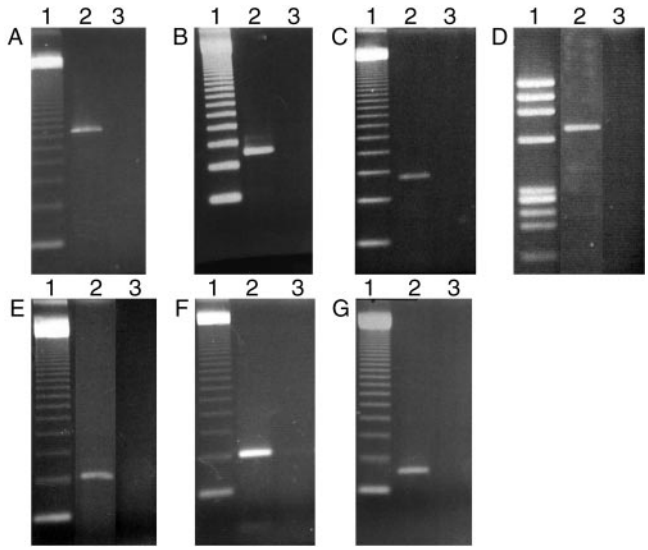

FIGURE 4. Agarose gel electrophoresis of RT-PCR products prepared in reactions RNA from rabbit corneal stromal cells with primers specific for the genes of the NADPH oxidase complex: (**A**) NOX4, 797 bp; (**B**) $p22^{phox}$, 316 bp; (**C**) $p47^{phox}$, 349 bp; (**D**) $p67^{phox}$, 714 bp; (**E**) $p40^{phox,}$ 249 bp; (**F**) Rac2, 252 bp; (**G**) GAPDH, 196 bp. *Lane 1*: molecular weight standard; *lane 2*: +RT; *lane 3*: −RT.

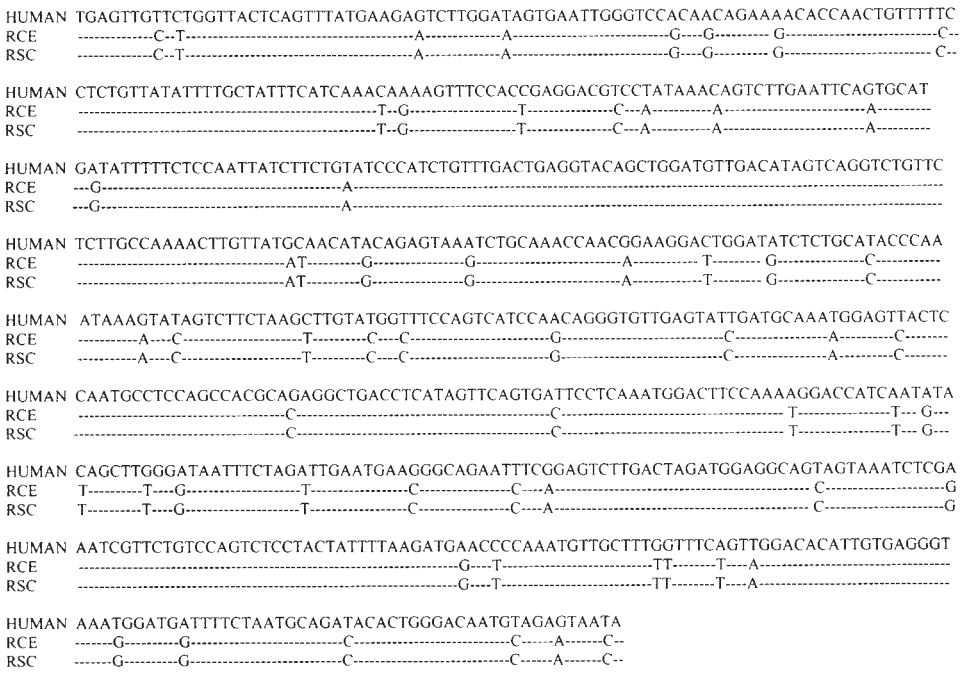

FIGURE 5. Sequence alignment of the NOX4 RT-PCR products from rabbit corneal epithelial (RCE) and stromal (RSC) cells with the human NOX4 sequence of cardiac myofibroblasts (AF254621).

control HL60 extracts (Figs. 7A, 7B). The broad band of protein observed in the region of 91 kDa was typical of gp91^{phox} in various states of maturation and glycosylation.[11] NOX2 protein was detected in extracts of rabbit neutrophils by these antibodies; thus, we know that if the protein were present, we would have expected to detect it. The protein of approximately 65 kDa detected in extracts of HL60 cells by the gp91^{phox} antibodies probably represents the precursor form of gp91^{phox}.[11] The NOX2 antibodies also detected a protein in the 65- to 66-kDa range in corneal epithelial and stromal cells but no protein was detectable in the 91-kDa region. The 65-kDa protein in stroma and epithelial cells could be a precursor of gp91^{phox} or another form of NOX, since the gp91^{phox} antibody (54.1) is known to bind other isoforms of NOX (Al Jesaitis, personal communications, April 2004).

NOX1 expression was not detected in either rabbit corneal epithelial or stromal cells with RT-PCR primers based on the human NOX1 sequence (AJ438989).[33] RNA extracted from rabbit lung cells, which would be expected to express NOX1, served as a positive control for these primers and produced a 522-bp product of the correct sequence from RNA extracted from these positive controls. The antibody to NOX1 recognized a protein of approximately 66 kDa in extracts of both rabbit corneal epithelial and stromal cells (Fig. 7D). Antibodies to NOX1 detected no protein in extracts from HL60 cells, as would be expected. It appears that the antibody to NOX1 recognized an epitope in NOX4, since the NOX1 antibody also reacted with a protein of approximately 66-kDa in HEK293 cells which express only NOX4. Thus, it appears that the functional NADPH complex in corneal epithelial and stromal cells is an NOX4- and is not an NOX1- or -2-based complex.

p22^{phox} associates with an NOX protein to form what is known as the cytochrome b_{558} complex.[11] The complex of these two proteins is necessary for the production of $O_2^{\cdot-}$ by the NADPH oxidase complex. Primers used to detect p22^{phox} transcripts were designed based on a human neutrophil cDNA sequence XM_008040, which has a high degree of identity

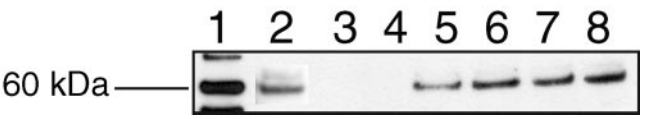

FIGURE 6. Western blot using an NOX4-specific antibody. The goat polyclonal antibody was prepared against a peptide of human NOX 4. Cells were grown, harvested, lysed, and fractionated by centrifugation at 29,000*g*. The pellets were suspended in buffer and subjected to Western blot analysis. Proteins contained in lysates are *lane 1*: molecular weight standards; *lane 2*: HEK293 cells; *lane 3*: HL60+DMSO; *lane 4*: HL60 untreated; *lanes 5* and *6*: rabbit corneal epithelial cells, *lanes 7* and *8*: RSCs.

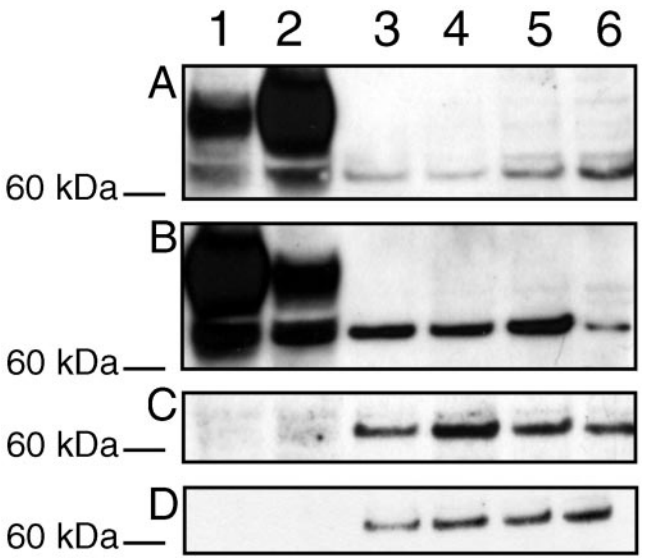

FIGURE 7. Western blot analysis with antibodies to detect the NADPH oxidase NOX proteins. Cells were grown, harvested, and lysed and the lysates separated by centrifugation at 29,000*g*. The pellet was suspended in buffer and boiled and the proteins separated on a 4% to 12% gel. Proteins were detected by blotting with antibodies to (**A**) gp91^{phox} (BD Transduction Labs), (**B**) gp91^{phox} (from Al Jesaitis), (**C**) NOX4, and (**D**) MOX1 (NOX1). *Lane 1*: HL60 without DMSO; *lane 2*: HL60 with DMSO; *lanes 3* and *4*: rabbit corneal epithelial cells; and *lanes 5* and *6*: rabbit corneal stromal cells.

with the rabbit cDNA sequence AF323787.[22] These primers have been demonstrated to amplify a 316-bp sequence from human neutrophils and human fibroblasts.[34] The primers amplified a product of the correct size in RT-PCR reactions using RNAs extracted from both rabbit corneal epithelial and stromal cells (Figs. 3B, 4B; $n = 9$ each). The sequences of the cDNAs from both rabbit corneal epithelial and stromal cells were 100% identical with that from rabbit leukocytes (AF323787).

Western blot analysis of 29,000*g*-sedimented fractions of cell lysates from cultured rabbit corneal epithelial and stromal cells showed a protein of approximately 17 kDa that reacted with two polyclonal antibodies to $p22^{phox}$ (Fig. 8). A protein of 21 kDa was observed in lysates of HL60 cells, which agrees with the literature reports.[35] HL60 cells also contained a protein at approximately 17 kDa, which reacted with the antibodies to $p22^{phox}$. This 17-kDa protein was likely a proteolytic product derived of $p22^{phox}$ reported by others.[36] These same antibodies identified a protein of 21 kDa in neutrophils of rabbits, which agrees well with the theoretical size of 20.7 kDa for $p22^{phox}$ in rabbit neutrophils.[32] Competitive binding assays using the immunizing peptide to compete for binding of $p22^{phox}$ to the $p22^{phox}$ goat polyclonal antibody eliminated the binding of the 17-kDa peptide and the 22-kDa peptide in the positive controls and eliminated the binding of the $p22^{phox}$ antibody to the 17-kDa peptide in our corneal samples.

A third protein required for NADPH oxidase activity is $p47^{phox}$. Primers ($p47^{phoxR}$) designed in our laboratory were based on the sequence of rabbit leukocyte $p47^{phox}$ cDNA (AF324409). The sense $p47^{phoxR}$ primer was located 330 bases from the 5′ end of the cDNA from rabbit leukocytes (AF324409). The reverse primer, $p47^{phoxR\ AS2}$, was located 679 bases from the 5′ end of the cDNA. This primer pair produced a 349-bp product from both rabbit corneal epithelial and stromal cells (Figs. 3C, 4C; $n = 12$ each). The sequences of the RT-PCR products were 99.3% identical with those of the rabbit leukocyte (AF324409).

Western blot analysis using 29,000*g* sedimented cell fractions of rabbit epithelial and stromal cells demonstrated a protein in the range of 46 to 47 kDa (Fig. 9). In cells other than neutrophils, $p47^{phox}$ and its isoforms have been found to be phosphorylated. The extent of phosphorylation has been related to the role of $p47^{phox}$ in initiating assembly of the proteins into complexes and to the activity of the complexes.[37,38] Further studies are needed to examine the extent of phosphorylation and/or modification of $p47^{phox}$ in rabbit corneal cells and to determine how phosphorylation might influence $O_2^{\cdot-}$ production in corneal cells.

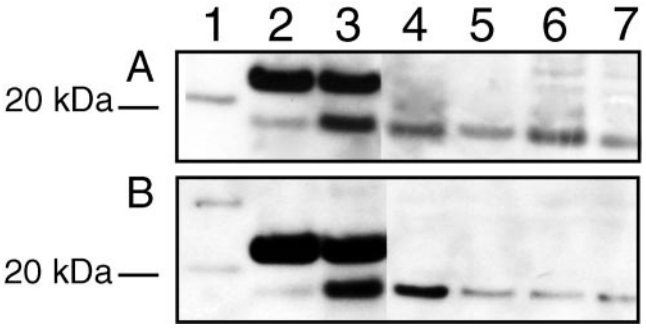

FIGURE 8. Western blot showing $p22^{phox}$ in rabbit corneal epithelial and stromal cells with (**A**) mouse polyclonal antibody to $p22^{phox}$ (44.1) and (**B**) a goat polyclonal antibody (Santa Cruz Biotechnology). *Lane 1*: molecular weight standard; *lane 2*: HL60+DMSO 29,000*g* sediment; *lane 3*: HL60 control 29,000*g* sediment; *lanes 4* and *5*: rabbit corneal stromal 29,000*g* sediment; and *lanes 6* and *7*: rabbit corneal epithelial 29,000*g* sediment.

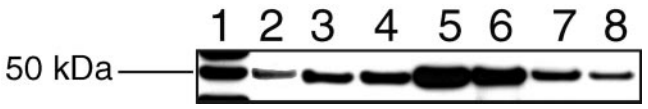

FIGURE 9. Western blot of proteins in the 29,000*g* pellet of rabbit corneal epithelial and stromal cells with an affinity-purified rabbit polyclonal antibody to $p47^{phox}$. *Lane 1*: molecular weight standard; *lane 2*: rabbit neutrophil lysate; *lane 3*: HL60+DMSO lysate; *lane 4*: HL60 control lysate; *lanes 5* and *6*: rabbit corneal stromal 29,000*g* sediment; *lanes 7* and *8*: rabbit corneal epithelial 29,000*g* sediment.

Two sets of RT-PCR primers were used to evaluate the expression of $p67^{phox}$, a fourth protein in the NADPH oxidase complex. One set of primers, designed based on a human neutrophil sequence (NM_000433), amplified an appropriate product from reactions with cDNAs generated from rabbit aortic fibroblasts and epithelial cells of the colon and, thus, served as a positive control.[18,23] These primers also amplified a 714-bp product from RNA of rabbit stromal cells (Fig. 4D). They did not amplify a product using RNA from rabbit corneal epithelial cells. The product amplified and sequenced from the stromal cell cDNA was 90.6% identical with the rabbit sequence AF323789 and 100% identical with the human sequence NM_000433. Thus, stromal cells express a $p67^{phox}$ message that is similar in composition to that of fibroblasts from other tissues.

Rabbit corneal epithelial cells produced $O_2^{\cdot-}$ by an NADPH oxidase-like mechanism, therefore, they should express $p67^{phox}$, because it is required for oxidase activity.[39,40] To detect $p67^{phox}$, we designed a second set of primers based on a rabbit neutrophil sequence (AF323789).[32] Amplification of rabbit corneal epithelial cell RNA by RT-PCR using this set of primers produced the predicted 363-bp product with a sequence that was 99.5% identical with the rabbit leukocyte sequence (Fig. 3D; $n = 9$). Amplification of RNA from rabbit stromal cells did not produce a product using these primers. Currently, it is unclear why the two corneal cell types express what appear to be different forms of $p67^{phox}$.

Western blot analysis prepared using $p67^{phox}$ antibodies (SC-7663; Santa Cruz Biotechnology, Inc.) documented the presence of a protein of 67 kDa in the membrane-containing fractions of the positive control HL60 cells treated with DMSO (Fig. 10). Blots containing proteins isolated from rabbit corneal epithelial and stromal cells had a dominant band at a slightly lower molecular mass (63–65 kDa; Fig. 10). Homologues of $p67^{phox}$, ranging in size between 51 and 73 kDa, have been reported.[18,41–43] Thus, despite the fact that the mRNAs produced by epithelial and stromal cells may be somewhat different, each appeared to produce a protein of similar size and reactivity (Fig. 10). At this time, it is unclear what differences exist between the $p67^{phox}$ gene products produced in rabbit corneal cells. Further studies are needed to characterize the differences in the $p67^{phox}$ proteins.

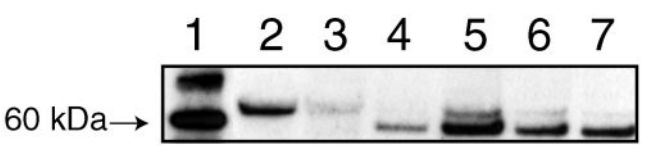

FIGURE 10. Western blot of proteins extracted from the 29,000*g* pellets prepared from lysed rabbit corneal epithelial and stromal cells. A goat polyclonal antibody to $p67^{phox}$ against an N-terminal domain of the human $p67^{phox}$ protein was used in the blot procedure. *Lane 1*: molecular weight standard; *lane 2*: HL60+DMSO; *lane 3*: HL60 control; *lanes 4* and *5*: rabbit corneal epithelial cells; *lanes 6* and *7*: rabbit corneal stromal cells.

The $p40^{phox}$ protein is not necessary for $O_2^{\cdot-}$ production, but it may play a role in regulating activity of the NADPH oxidase complex.[44] Two sets of primer pairs were designed based on the rabbit leukocyte sequence AF323790.[32] RNA extracted from rabbit corneal epithelial cells produced an RT-PCR product of the correct size with both primer pairs ($n = 9$ each). The products that primers $p40^{phoxR}$ and $p40^{phoxR2}$ produced from epithelial cell cDNAs were 98.8% and 99.5% identical with the rabbit leukocyte sequence, respectively. The product of the $p40^{phoxR}$ primer is shown in Figure 3E. Stromal cell RNA, however, produced product only with the $p40^{phoxR2}$ primer pair (Fig. 4E; $n = 12$). The sequence of the stromal cell product was 99.5% identical with that of rabbit leukocyte (AF323790) and 100% identical with the $p40^{phoxR2}$ product produced from epithelial cells. The fact that the epithelial cells produce RT-PCR products from both primers suggests that they make the full-length $p40^{phox}$ product similar to rabbit leukocytes. Stromal cells may produce a variant form of the protein that is very slightly different.

Western blot analyses of extracts from HL60 cells using $p40^{phox}$ antibodies demonstrated two bands of reactivity—one at 42 kDa and the other at 38 kDa—that served as positive controls (Fig. 11). This observation is consistent with the work of others who have demonstrated a doublet of proteins of approximately 42 kDa and 38 kDa.[45] The lower molecular size band is believed to be a proteolytic breakdown product of $p40^{phox}$ in HL60 cell extracts. A 39-kDa protein isolated from guinea pig cells is believed to be a $p40^{phox}$ homologue.[43] Western blot analysis of the proteins from both corneal epithelial and stromal cells demonstrated two bands of immunoreactivity as in the HL60 control, except that there was much less breakdown product in corneal cell preparations (Fig. 11). Western blot analysis demonstrated little if any difference between the proteins in epithelial and stroma cells, despite the differences in RT-PCR products. Further studies are needed to determine whether the two bands present in the Western blot analysis represent splice variants or breakdown products.

In addition to the unique proteins that compose the NADPH complex, the small GTP-binding protein Rac is necessary for $O_2^{\cdot-}$ production both in vivo and in vitro.[14] Few publications actually document the presence of Rac in corneal cells, and so we believed it was necessary to document the existence of a Rac protein in our cell cultures.[46] Primers to amplify Rac1, -2, and -3 mRNA were designed based on mouse Rac1 XM_132485, Rac2 on mouse NM_009008, and Rac3 on mouse AB040819. Alignments of each of the primers with each of the complete cDNA sequences of the three Rac isoforms indicated that the primers for MuRac1, -2, and -3 were sequence-specific and should not cross-amplify the other isoforms. Rabbit corneal epithelial cells expressed mRNA that could be amplified to give a 395-bp sequence that was 91.5% identical with that of mouse Rac 1 (Fig. 3F; $n = 6$). Epithelial cells did not produce an RT-PCR product using primers for Racs2 or -3. Stromal cells expressed mRNA that amplified with primers to Rac2, producing a 252-base sequence but no product was detected with primers to Rac1 or -3 (Fig. 4F; $n = 6$). Sequencing of the RT-PCR product of the stromal cell reactions with the Rac2 primers indicated that the product was 94.5% identical with the Rac2 sequence (NM_002872) of human monocytes and 88.2% identical with the mouse macrophage sequence (NM_009008; data not shown).

Western blot analysis with antibody that recognizes most isoforms of Rac produced an intense band at approximately 21 kDa in soluble and membrane preparations of rabbit corneal epithelial and stromal cells and in positive control HL60 cells (Fig. 12). Corneal epithelial cells contained a protein that reacted with the Rac1-specific antibody. Stromal cells constitutively produced a protein that reacted with the isoform-nonspecific Rac antibody but did not react with a Rac2-specific antibody. The data suggest that an isoform of Rac was produced in rabbit stromal cells and that it associates with the particulate fraction along with the NADPH oxidase, but it is unclear which isoform of Rac is expressed. The data reported herein are the first to confirm Rac expression in corneal cells. The role of Rac in the production of superoxide by NADPH oxidase in corneal epithelial and stromal cells remains to be determined.

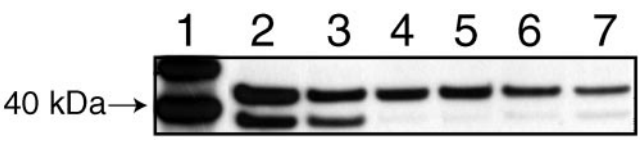

FIGURE 11. Western blot analysis of proteins extracted from the fraction of rabbit corneal epithelial and stromal cells that sedimented at 29,000*g*. Blots were prepared with a monoclonal antibody to a human $p40^{phox}$. *Lane 1*: molecular weight standard; *lane 2*: HL60+DMSO; *lane 3:* HL60 control; *lanes 4* and *5*: rabbit corneal stromal cells; *lanes 6* and *7*: rabbit corneal epithelial cells.

DISCUSSION

Production of $O_2^{\cdot-}$ by NADPH oxidase complexes has been considered principally as a neutrophil-specific mechanism of defense against pathogens. The neutrophil oxidative burst has been known to exist for many years, but the details of the enzymology have been extensively characterized within the past 10 years.[47] Cells other than neutrophils are also capable of producing $O_2^{\cdot-}$ by means of an NADPH oxidase system, albeit in smaller amounts. The complexes, as they exist in cells that are not of myeloid origin, have several differences from those found in neutrophils, including the rate of $O_2^{\cdot-}$ production, the isoforms of the component proteins, the regulatory properties of the complex's activities, and the intracellular localization.[6] The primary function of the neutrophil complex is to produce large amounts of $O_2^{\cdot-}$ rapidly to react with halides and other molecules forming highly reactive oxidative molecules necessary to kill bacteria and other pathogens. It is less clear why cells other than neutrophils produce $O_2^{\cdot-}$. The free-radical theory of aging posits that $O_2^{\cdot-}$ and other radicals like OH• are formed by the reaction of single electrons escaping from the mitochondrial electron transport and these radicals damage or age tissues.[48,49] It is now clear, however, that $O_2^{\cdot-}$ and other radicals of various forms play critical roles in many cellular functions. In mesangial cells, $O_2^{\cdot-}$ generated from an NOX4-based NADPH oxidase complex functions as a mediator of signal transduction of angiotensin II.[50] In vascular endothelial cells, $O_2^{\cdot-}$ generated by the NADPH oxidase may involve VEGF signaling.[51] In kidney cells, $O_2^{\cdot-}$ produced by NADPH oxidase may function as an oxygen sensor and/or a regulator of cell growth.[21] The tryosyl radical present in the enzyme ribonucleotide reductase plays an essential role in catalysis.[52] Thus, free radicals and other radicals play significant roles in functions critical to cell survival.

The production of $O_2^{\cdot-}$ in nonmyeloid cells may occur through any of several pathways depending on the status of the cell. It appears that rabbit corneal epithelial and stromal cells constitutively produce $O_2^{\cdot-}$ in vivo and in vitro. Human and rabbit corneal cells possess xanthine oxidoreductase which is capable of producing $O_2^{\cdot-}$ under certain conditions.[53,54] Corneal cells also contain other enzymes, such as nitric oxide synthase, and electron transport systems, such as mitochondrial electron transport, that are capable of producing $O_2^{\cdot-}$ under conditions of stress, but it unclear how often $O_2^{\cdot-}$ is produced under physiological conditions $O_2^{\cdot-}$.[3,4,10]

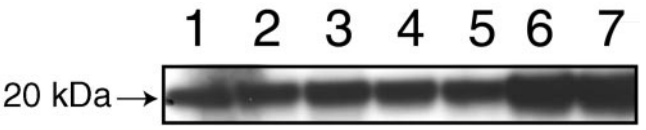

FIGURE 12. Western blot analysis detecting the expression of Rac in fractions of rabbit corneal cells sedimented at 29,000*g*. The antibody to Rac was a non–isoform-specific antibody. *Lane 1*: molecular weight standard; *lane 2*: HL60+DMSO; *lane 3*: HL60 control; *lanes 4* and *5*: rabbit corneal stromal cells; *lane 6* and *7*: rabbit corneal epithelial cells.

The data presented herein document that both rabbit corneal epithelial and stromal cells contain NADPH oxidase complexes, which constitutively produces $O_2^{\cdot-}$ from NADPH. The specific activity of the NADPH oxidase, as we have measured it, is in the range of other nonmyeloid cells (1–4 nanomoles/min per milligram protein), an amount 10 to 100 times less than that of the respiratory burst of neutrophils.[25,28] The use of EPR to analyze adducts formed during the reaction of $O_2^{\cdot-}$ with BMPO produced hyperfine splitting constants that confirm that $O_2^{\cdot-}$ was the product of the oxidase reaction.[26] The data further document that the $O_2^{\cdot-}$ produced was unlikely to have as its source nitric oxide synthase, xanthine oxidase, or mitochondrial electron transport. At this time, no absolutely specific inhibitors of NADPH oxidase are known but the fact that the production of $O_2^{\cdot-}$ was NADPH dependent, associated with the particulate fraction, and inhibited by DPI suggests that NADPH oxidase is the source. The fact that the NADH and succinate were not good substrates and that specific inhibitors such as 1400W, L-NAME, rotenone, azide, and allopurinol were not effective inhibitors helps confirm that the production of $O_2^{\cdot-}$ from the cell preparations was due to NADPH oxidase.

RT-PCR and Western blot analysis were used to confirm that rabbit corneal epithelial and stromal cells produce mRNA and proteins consistent with NADPH oxidase complexes. We have determined that the complexes, as they exist in these corneal cells, contain the NOX 4, $p22^{phox}$, $p47^{phox}$, $p40^{phox}$, and an isoform of $p67^{phox}$. The cells also express the required regulatory protein Rac.

NOX 4 is an isoform of NOX found in other cells that are not of myeloid origin such as kidney epithelial cells, vascular endothelial cells, and adventitial fibroblasts.[21,55] Little is known about the mechanism(s) by which NOX 4 may influence the function of the complex. It has been proposed that NOX4 and the other NOX isoforms may be responsible for the turnover number and regulation of the complex activity. NOX4-containing complexes are capable of (1) mediating angiotensin II-induced activation of Akt in mesangial cells, (2) mediating in conjunction with TLR4 the response to LPS, and (3) influencing the response of blood vessels to pressure overload.[50,56,57] We are in the process of determining the function of the NOX 4 complex in corneal cells.

$p22^{phox}$ was present in rabbit corneal cells as documented by RT-PCR and Western blot analysis with two different antibodies. The mRNA detected by RT-PCR was produced constitutively in both epithelial and stromal cells. The sequence of the product was 100% identical with that produced by rabbit neutrophils. $p22^{phox}$ is essential for activity and is believed to contain the membrane-spanning helices and a docking site for $p47^{phox}$.[58,59] Only on occasion did we isolate it in the 21.9-kDa form. This protein is highly susceptible to proteolysis. A 17-kDa component of the spectrally stable proteolytic fragment of flavochrome b has previously been isolated.[36] We have confirmed that the 17-kDa protein in our Western blot analysis was a fragment of $p22^{phox}$ through competitive binding studies by employing the peptide used to prepare one of the antibodies to $p22^{phox}$ as a competitor.

A third protein of the complex is $p47^{phox}$. RT-PCR reactions using RNAs extracted from both rabbit corneal epithelial and stroma cells amplified 349-bp products that were 99.3% identical with that produced from rabbit leukocytes. Western blot analysis confirmed the production of a protein of approximately 47 kDa, which probably represents $p47^{phox}$. $p47^{phox}$ is believed to play roles in the assembly and activity of the NADPH oxidase complex. $p47^{phox}$ contains both SH3 domains and PX domains. The PX domains influence direct binding to phosphoinositides (PIs) of membranes.[60] The SH3 domains provide multiple phosphorylation sites that are thought to influence the activity of the NADPH complex and the localization of $p47^{phox}$.[60] It is hypothesized that phosphorylation of the SH3 domains permits a conformational change to occur that exposes the PX domains, thus allowing binding of $p47^{phox}$ to PIs in the membrane and interaction with $p22^{phox}$.[12] Phosphorylated forms of $p47^{phox}$ and its homologues function as organizers of the complex and may influence the isoform of NOX with which the complex associates.[41] In neutrophils, PI3 kinase-dependent phosphorylation of $p47^{phox}$ by protein kinase B (Akt) influences the amount of $O_2^{\cdot-}$ produced.[61] Also, cytokines such as TNF-α induce rapid and limited phosphorylation of specific tyrosine residues resulting in enhanced $O_2^{\cdot-}$ production.[37]

A fourth protein that is necessary for activity of the NADPH oxidase complex is $p67^{phox}$.[41] $p67^{phox}$ and its homologues appear to be activators of the NADPH complex.[30] The activator function of $p67^{phox}$ and its homologues regulate the rate-limiting hydride transfer from NADPH to FAD in cytochrome b_{558}. RT-PCR reactions using RNA from rabbit corneal epithelial cells amplified a 363-bp product that was 99.5% identical with that from rabbit neutrophils. These primers did not amplify a product from the cDNA of rabbit stromal cells. However, when primers that were designed based on the human neutrophil sequence were used to amplify the cDNA prepared from stromal cells, a product of 714 bp was produced that was 100% identical with the human neutrophil sequence. Western blot analysis documented the presence of a 67-kDa protein in the positive control cells. Both rabbit epithelial and stromal cells produced a protein in the 63- to 65-kDa range that strongly reacted with the $p67^{phox}$ antibody. These data are consistent with the size of the isoforms of $p67^{phox}$ found in guinea pig but not rabbit neutrophils.[32,62] In cells that are not of myeloid origin, $p67^{phox}$ homologues exist with molecular weights as low as approximately 51 kDa.[41]

The fifth protein in the complex is $p40^{phox}$. Controversy exists regarding the need to have $p40^{phox}$ in the complex, because in some cells it is not necessary for activity. It is believed that the state of phosphorylation may influence the rate or amount of $O_2^{\cdot-}$ produced.[45,63] $p40^{phox}$ may function both as a positive or a negative regulator of activity.[64] The form of $p40^{phox}$ containing phosphorylated threonine 154 exerts a negative regulatory affect while the unphosphorylated form is a positive regulator of activity.[65] $p40^{phox}$ also appears to stabilize the complex.[66,67] RT-PCR using RNA from rabbit corneal epithelial cells and either of two sets of primers produced products that were 98.8% and 99.5% identical with the rabbit leukocyte sequence. Stromal cell RNAs produced an RT-PCR product only from one set of primers, so there must be some difference in the sequences of the epithelial and stromal $p40^{phox}$. The stromal cell RT-PCR product was 99.5% identical with the product produced from the rabbit leukocytes. This product was 100% identical with the product produced from corneal epithelial cell RNA by the same primers. Western blot analysis demonstrated a clear dominant band of protein at 42 kDa from both cell types and a weaker band at 38 kDa. The 38-kDa band is believed to be a breakdown product of the

42-kDa form. The molecular weights correspond well with the actual and predicted molecular weights of rabbit $p40^{phox}$.[32,45]

The small GTP-binding protein Rac is necessary for activity and appears to associate with the complex of proteins in the particulate fraction.[14] Only one report alludes to the presence of Rac in corneal cells,[46] so we believed that it was necessary to document its presence in our cultured corneal cells. Based on RT-PCR, Rac 1 mRNA was expressed in corneal epithelial cells, but no indication of the expression of Rac2 or -3 was observed. Western blot analysis confirmed that rabbit corneal epithelial cells expressed Rac. Some cell types such as neutrophils express more than one isoform of Rac. Human neutrophils, for example express both Rac1 and -2, but Rac2 accounts for 96% of the Rac protein.[15] Stromal cells appeared to express Rac 2 mRNA, however no protein was detected with Rac 2-specific antibody. Western blot analysis with the Rac2 specific antibody detected a protein of approximately 21 kDa in the control HL60 cells so the antibody seemed specific. Thus, it is likely that the NADPH oxidase of rabbit epithelial cells uses the Rac1 present in the particulate fraction to regulate NADPH oxidase activity. Stromal cells express a form of Rac that reacts with the isoform nonspecific antibody, but differs from Rac1 in the region amplified by our RT-PCR primers. It is unclear how stromal cell Rac interacts with the oxidase complex. Further studies are needed to characterize the Rac isoforms present in rabbit corneal stromal cells and to determine the nature of interactions with the NADPH oxidase.

In summary, our data document constitutive expression of the genes and the production of the five core proteins necessary to form an active NADPH oxidase complex by cultured rabbit corneal epithelial and stromal cells. Superoxide was produced by both cell types at a rate of 1 to 4 nanomoles/min per milligram in an NADPH- but not NADH-dependent manner. In both cell types, the complex was composed of NOX4, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$. Rac1, a regulator of superoxide production by NADPH oxidase was expressed in rabbit corneal epithelial cells. A variant form of Rac appeared to be expressed in rabbit corneal stromal cells. The regulation of the activity and the function of the superoxide produced remains to be determined. Thus $O_2^{\cdot-}$ produced in rabbit corneal epithelial and stromal cells needs to be considered as a regulator of cellular functions in normal as well as in inflamed corneas.

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