The Regulation of NADPH Oxidase and Its Association with Cell Proliferation in Human Lens Epithelial Cells

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\textbf{PURPOSE.} NADPH oxidase (NOX)–generated reactive oxygen species (ROS) are essential for growth factor–stimulated cell proliferation. In this study, the regulatory role of p22phox, a membrane subunit of NOX, in NOX activity and platelet-derived growth factor (PDGF) mitogenic signaling were examined.

\textbf{METHODS.} Human lens epithelial B3 (HLE B3) cell lines with p22phox overexpressed (p22-OE) and p22phox knockdown (p22-KD) were used as models. Cells stimulated with PDGF were compared with nonstimulated control cells. The relative NOX activity and intracellular ROS generation were detected by lucigenin-based assay and DCFH fluorescence, respectively. Cell proliferation was measured by BrdU and fluorescent nuclear staining.

\textbf{RESULTS.} p22-OE showed higher NOX activity, PDGF-stimulated ROS generation, cell proliferation, and activation of signaling cascades of ERK1/2, JNK, and Akt over the control (vector alone). In contrast, p22-KD showed opposite results. In addition, PDGF stimulated p47phox and Rac1 translocations and induced binding between p22phox and the cytosolic subunits of p47phox, p67phox, and p40phox. Overexpression of p22phox increased p22phox-p47phox binding, enhanced cell proliferation, and prolonged the phosphorylation of PDGF receptor at Tyr857 with a corresponding inhibition of the activity of the downstream targets Src, MAPK ERK1/2, and p38 MAPK.

\textbf{CONCLUSIONS.} PDGF mitogenic action in HLE B3 cells depends on p22phox to regulate NOX activity, which affects PDGF receptor function for cell proliferation. (Invest Ophthalmol Vis Sci. \textbf{2009;50:2291–2300}) DOI:10.1167/iovs.08-2568

\textbf{Platelet-derived growth factor (PDGF) along with several other growth factors plays a critical role in human lens growth and development.\textsuperscript{1,2} The lens epithelial cells, which are rich in receptors for PDGF and other growth factors, continuously proliferate and differentiate into lens fiber cells during normal lens development and throughout life.\textsuperscript{3} For instance, it has been observed that pulsatile delivery of PDGF into culture medium induces growth of rat lens in culture and that PDGF is necessary for maintaining lens transparency.\textsuperscript{4} Although reactive oxygen species (ROS) are known to be harmful substances,\textsuperscript{5} the recent findings that ROS generated from NOX are essential mediators for growth factor–or cytokine-induced cellular functions suggest a new physiological role of ROS in many nonphagocytic cells.\textsuperscript{6–9} Using human lens epithelial B3 cells (HLE B3) as a model, we observed that PDGF-induced signaling for cell proliferation is mediated by ROS and that certain level of ROS, either generated in situ or provided externally, is necessary for maintaining normal cell growth.\textsuperscript{10} Furthermore, PDGF-induced arachidonic acid (AA) release from cell membrane appears to be an upstream key step that controls ROS generation,\textsuperscript{11} whereas PDGF receptor and its target proteins of Src, small GTPase Rac, and Ras all play an essential regulatory role in redox signaling.\textsuperscript{12} Thus, we have proposed a mechanistic pathway for ROS-mediated PDGF signaling, in which the process involves an initial activation of PDGF receptor, followed by downstream phosphorylation of Src, MAPK ERK1/2, and the subsequent activation and translocation of cytosolic phospholipase A\textsubscript{2} (cPLA\textsubscript{2}) for AA release from the cell membrane.\textsuperscript{10,11} Our evidence suggests that AA and its metabolites act as a positive feedback loop for further ERK1/2 activation and mitogenic signaling in the nucleus by stimulating NADPH oxidase to generate ROS in lens epithelial cells.\textsuperscript{11}

NADPH oxidase (NOX) is known to be an essential enzyme in phagocytes to generate ROS for host defense.\textsuperscript{3} Extensive evidence has now shown that NOX is also one of the major sources for ROS production in various nonphagocytic cells, including smooth muscle cells,\textsuperscript{5} kidney proximal tubular cells,\textsuperscript{13} hepatocytes,\textsuperscript{14} and lens epithelial cells.\textsuperscript{10,16} These findings revealed a new physiological role of NOX in regulating redox signaling for cell proliferation and other functions.

The catalytic subunit in NOX is commonly present in several homologues in the mammalian cells, including NOX1, -2 (gp91phox), -3, -4, and -5 plus the thyroid-specific DUOX1 and -2.\textsuperscript{17} In the case of NOX2, which is most widely distributed among all the NOX isozymes, its activation requires the assembly of various subunits,\textsuperscript{18} including the membrane-bound gp91- and p22phox, and the cytosolic components of p47phox (the organizer), p67phox (the activator), Rac1/2, and p40phox.\textsuperscript{19–21} The activation process of NOX involves the formation of a complex between gp91- and p22phox, called cytochrome b558, followed by using this complex as a docking site to assemble the phosphorylated and activated p47phox, which organizes the translocation of p67- and p40phox and allows p67phox to interact and activate gp91phox.\textsuperscript{22,25} In addition, the small GTPase Rac1/2 plays a role in NOX activation via its direct interaction with gp91phox.\textsuperscript{24} Other NOX homologues of NOX1, -3, or -4 are also p22phox-dependent but NOX1 and -3 use NOX1A1 (homologue of p47phox) to organize the enzyme assembly and NOXO1 (homologue of p67phox) for NOX activation.\textsuperscript{5,17} NOX4 is p22phox-dependent but does not require any cytosolic subunit for activation, whereas both NOX5 and DUOX1/2 are independent of...
p22phox and other subunits and only use calcium for activation.5,17

Rao et al.16 first reported that all the components of the NOX2 system were present in the lenses of various animal species and that they were distributed mainly in the epithelium with very low levels in the cortex or nucleus. However, although the authors found p22phox or gp91phox mRNA, they did not examine whether p22phox or gp91phox proteins are present in the human lens epithelial cells.16 Thus, whether NOX2 is functional in cell proliferation in the lens or how NOX2 activity is regulated in the lens remains a question. Of the subunits of NOX2, p22phox has been indicated as a key factor in regulating NOX enzyme function25–27; however, the molecular mechanisms on the activation and the regulation of NOX in the mammalian cells are still not fully understood. Therefore, we investigated the presence of p22phox and gp91phox proteins in the human lens tissue and lens epithelial cells. We also examined the regulatory role of p22phox in NOX2 activity, using p22phox knockdown and overexpressed cells. We also examined the regulatory role of p22phox in controlling PDGF or AA-induced mitogenic signaling in the human lens epithelial cells. For simplicity, NOX2 will be referred as NOX throughout the paper, unless otherwise noted.

MATERIALS AND METHODS

Eagle’s minimum essential medium (EMEM), fetal bovine serum (FBS), and 2′,7′-dichloro-fluorescin diacetate (DCF-DA) were purchased from Invitrogen Corp. (Carlsbad, CA); p-nitrophenyl phosphate [pNPP], HEPES, trypsin, AA, lucigenin, β-glycerophosphate, chymostatin, 3,4-DCl, E-64, leupeptin, pepstatin A, phenylmethylsulfonl fluoride (PMSF), N-acetylcyesteine (NAC), and NaVO₃ from Sigma-Aldrich (St. Louis, MO); and antibodies against phospho-p12/44 MAPK (ERK1/2), phospho-JNK, phospho-p38, phospho-Akt, and Rac1 from Cell Signaling Technology Inc. (Beverly, MA). Antibodies against glyceraldehyde-3-phosphate dehydrogenase (G3PD); p22phox, p47phox, p67phox, and Rac1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The enhanced chemiluminescent (ECL) system components were from Pierce Biotechnology (Rockford, IL). Nitrocellulose membrane (Hybond-ECL) was from Amersham Biosciences Inc. (Piscataway, NJ).

Preparation of Human Lens Homogenate

Human donor eyes (one pair from a 65-year-old male) without pathologic conditions were obtained from the Omaha Lions Eye Bank (Omaha, NE) less than 24 hours after death. The lenses were removed from the fresh eyes and stored at −80°C and used within 2 weeks. The center of the partially thawed lens was removed with a 7-mm steel cork borer. After the two ends of 3 mm were cut off, the remaining tissue of the center cylinder was considered the nucleus (30% of the total lens weight). The two ends and the balance of the lens were pooled and considered the epithelium-cortex. Each lens portion was homogenized in 2 ml pH 7.4 phosphate buffer with a glass homogenizer, followed by centrifugation at 10,000 rpm for 30 minutes at 4°C before the supernatant was used for protein measurement and Western blot analysis for p22- and gp91phox.

Cell Culture

Human lens epithelium (HLE) B3 cells were a gift from Usha Andley (Washington University, St. Louis, MO). The cells were cultured in MEM containing 20% fetal bovine serum (FBS) until reaching confluency, after which the cells were gradually deprived of serum by first culturing in the medium with 2% FBS overnight followed by culturing in serum-free medium for 24 hours before further experimental use.

Primary mouse lens cells prepared in our laboratory28 was cultured as just described and used as controls for HLE B3 cells. The mouse cells were analyzed both for NOX activity and for PDGF-induced ROS production as described in the following sections.

Overexpression and Knockdown of NOX Subunit p22phox in HLE B3 Cells

Total RNA was extracted from HLE B3 cells by using an SV total RNA isolation kit (Promega, Madison, WI). cDNA was obtained by applying in vitro reverse transcription. PCR was performed with specific primers: 5′-CACATGGGGCAGATCGAGTGGG-3′ and 5′-CACACGAACCTCCTGCGTACAC-3′. The size of p22phox DNA was determined by electrophoresis, and the p22phox DNA was cloned into the pCR3.1 expression vector (Invitrogen) by using restriction endonuclease digestion and ligation. The expression vector carrying inversely oriented p22phox was used to generate antisense RNA for the knockdown experiment and the vector with normally oriented p22phox was used for the overexpression experiment. The expression vector was introduced into HLE-B3 cells by electroporation. The mixed and stable transfected cell line was obtained after geneticin (G418, 1.0 mg/mL) selection for 4 weeks. The p22phox knockdown cells (p22-KD), p22phox overexpression cells (p22-OE), and the cells with vector alone (Vec, control) were stored in −80°C pending experimental use.

NOX Activity Assay

NOX was assayed with lucigenin used as the substrate, according to the method of Zhang et al.31 with some modifications. Lucigenin is a luminescence-generating reagent that interacts with free radicals such as superoxide anion and the emitted luminescence can be quantitatively recorded and analyzed by a luminescence detector. Since NOX is a major source of free radicals in the nonphagocytic cells, the emitted luminescence reflects its relative activity. In short, cells (1 × 10⁶) were washed thoroughly with PBS and disrupted by sonication in ice-cold Krebs buffer of pH 7.4 (130 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 1.5 mM CaCl₂, 5 mM glucose, 35 mM phosphoric acid, and 20 mM HEPES). The cell homogenate was centrifuged at 1000g, and the pellet was resuspended with luminescence buffer (Krebs buffer containing 0.5 mM lucigenin), before adding NADPH (0.1 mM) as the substrate. The emitted luminescence was detected by a luminescence detector (FluoStar Optima; BMG Labtech, Durham, NC). Typically, the readings of the luminescence increased linearly within 5 minutes, and the slope for the trend line was defined as the relative NOX activity, expressed as luminescence unit (RLU)/minute. Primary mouse lens epithelial cells were prepared and analyzed for NOX activity with and without PDGF (20 ng/mL) treatment, as described for the HLE B3 cells.

Analysis of Cell Proliferation

Nucleic Acid Staining. HLE B3 cells (2 × 10⁴ cells/well) were seeded in 96-well plate. After incubation for 24, 48, and 72 hours, cells were lysed in 200 μL TE buffer containing 0.1% SDS, and mixed with green nucleic acid dye (PicoGreen; Invitrogen), according to the manufacturer’s guidance. The excitation/emission of the dye was 485/520 nm, and the relative fluorescence intensities were detected by the luminescence detector (FluoStar Optima; BMG Labtech).

Bromodeoxyuridine Staining. HLE B3 cells (8 × 10⁴ cells/well) were cultured on a 96-well plate and serum-free starved for 24 hours. The cells were then incubated in MEM containing 20 ng/mL PDGF or 60 μM AA, or 20% FBS for 30 minutes. After incubation, the medium in each well was replaced with MEM containing BrdU from a cell proliferation assay kit (Chemiluminescence Assay; Roche Applied Science, Indianapolis, IN) and the cells were incubated at 37°C for 4 hours followed by the chemiluminescent immunoassay for quantifying cell proliferation, used according to the manufacturer’s instructions.

Analysis of Cell Apoptosis

Cells (2 × 10⁴) of each transfected (p22-KD, p22-OE, and Vec) were trypsinized and then washed with PBS. The apoptosis levels of the cells...
were detected by using an Annexin V apoptosis detection kit (Biovision, Mountain View, CA) and flow cytometry, according to manufacturer’s protocol. This method detects the translocation of phospholipid phosphatidylserine from the inner surface of the plasma membrane to the outer surface of the cell, which signifies the occurrence of apoptosis. Some of the cells were treated with 150 μM of H_{2}O_{2} for 1 hour to induce apoptosis as the positive control. After H_{2}O_{2} treatment, the cells were incubated in MEM containing 20% FBS for 16 hours before the apoptosis analysis.

**Measurement of ROS Generation Stimulated by PDGF, AA, or FBS in HLE B3 Cells**

HLE B3 cells transfected with p22phox overexpression (p22-OE) or p22phox knockdown (p22-KD) expression plasmid were cultured on a 96-well plate (2 × 10^{4} cells/well) and serum starved for 24 hours. The cells were then incubated in MEM-containing the fluorescent dye DCF-DA (50 μM) for 5 minutes in the dark, washed thoroughly by MEM and further incubated in MEM, with or without 20 ng/mL PDGF, or 60 μM AA, or 20% FBS. The emitted fluorescence was measured by luminometer (FluoStar Optima; BMG Labtech), with excitation and emission wavelengths at 485 and 520 nm, respectively. Mouse primary lens epithelial cells were prepared and analyzed for PDGF (20 ng/mL)-stimulated ROS production according to the same procedures used for HLE B3 cells. Some of the cells were pretreated with 10 or 40 mM NAC for comparison.

**Translocation of p47phox in Cells Stimulated by PDGF**

HLE B3 cells (5 × 10^{6}) were washed thoroughly with ice-cold PBS, incubated in lysis buffer (containing 50 mM Tris-HCl [pH 7.4]; 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 6 μg/mL chymostatin, 10 μM 3,4-DCl, 10 μM E6-4, 1 μg/mL leupeptin, 1 μM pepstatin A, 10 μM PMSE, and 200 μM Na_{2}VO_{4}), and then scraped off the culture plate, followed by sonication. The cell homogenate was centrifuged at 1500 g to remove the cell debris, and the supernatant was centrifuged at 100,000 g for 20 minutes. The supernatant (cytosol portion) was reserved for future use while the pellet was resuspended with the same lysis buffer, but containing 1% Triton X-100 for membrane isolation. After centrifugation at 100,000 g for 20 minutes, the supernatant (membrane portion) was saved. Both the cytosolic and membrane portions were subjected to Western blot analysis with specific anti-p47phox antibody (Santa Cruz Biotechnology).

**Immunoprecipitation of p22phox**

Cells (4 × 10^{8}) were washed twice with ice-cold PBS and lysed in 700 μL RIPA buffer by sonication. The cell debris was removed by centrifugation at 1000g for 5 minutes. Cell lysate (200 μg protein) was mixed with 5 μg anti-p22phox antibody (Santa Cruz Biotechnology) by gentle rocking at 4°C for 16 hours and then mixed with 50 μL of protein-agarose beads (A/G Plus; Santa Cruz Biotechnology), followed by gentle rocking at 4°C for another 2 hours. The beads were collected by centrifugation at 1000g, washed gently three times with PBS, and mixed with SDS-PAGE protein loading buffer (200 μL). The mixture was boiled for 5 minutes and centrifuged (10,000g for 5 minutes), and the supernatant was saved for future use.

**Western Blot Analysis**

Cells (1.3 × 10^{6}) were washed twice with ice-cold PBS and lysed in 500 μL lysis buffer, as described earlier. The soluble proteins (20 μg/well) were separated by 10% SDS-PAGE, and the gel was electroblotted onto nitrocellulose membrane (Hybond-ECL; Amersham) and then probed with anti PERK1/2, P-JNK, P-P38, and P-Akt. The Pan PTP activity was analyzed by using pNPP as the substrate, according to the method of Xu et al.29 Because there is no direct assay method available for LMW-PTP, this enzyme was immunoprecipitated before reacting with pNPP substrate for the activity assay. In short, LMW-PTP in the cell lysate was immunoprecipitated by specific anti-LMW-PTP antibodies and protein A/G-conjugated beads (Santa Cruz). The enzymatic reaction was performed in 0.1 M acetate buffer (pH 5.5) containing 5 mM pNPP and incubated at 37°C for 30 minutes and was terminated by 100 μL of 1 N NaOH. The absorbance of the final product in the solution was read at 405 nm by a spectrophotometer. The relative enzyme activities were compared according to the changes in absorbance readings.

**Protein Concentration Determination and Statistical Analysis**

Protein concentration was determined by the BCA method.30 Statistical data were analyzed by analysis of variance (ANOVA). For all tests, P < 0.05 was considered significant. The error bars in all pixel intensity analyses of the Western blot represent standard deviations.

**RESULTS**

To verify whether p22phox and gp91phox are expressed in the human lens tissue, we used donor human lenses dissected into epithelium-cortex and nuclear fractions and examined by Western blot analysis with anti-p22phox and anti-gp91phox antibodies, respectively. Figure 1A shows that gp91phox was detected in both the nuclear and epithelium-cortex fractions, whereas p22phox showed high levels in the epithelium-cortex and was absent in the nuclear fraction.

**Regulation of NADPH Oxidase and PDGF Signaling**

By using the primers flanking human p22phox, we obtained a single DNA band with 582 bp (Fig. 2A) from the total RNA of HLE B3 cells by RT-PCR. The sequence of this 582-bp DNA was found to be identical with human p22phox (GenBank:
and activate a signaling cascade in human lens epithelial cells. Therefore, we investigated the association of p22phox expression level with ROS generation in cells stimulated by PDGF or AA. All three transfectants (p22-KD, p22-OE, and Vec) were treated with PDGF (20 ng/mL), AA (60 μM), or 20% serum for 10 minutes, and the ROS generated were analyzed by DCF-DA-based fluorescence assay. Quiescent cells (without stimulation) displayed a similar level of fluorescence or ROS in both Vec and p22-OE cells, whereas the ROS level in p22-KD cells was 20% less (Fig. 3B). When the cells were stimulated by PDGF, the ROS level increased 20% to 30% in the Vec cells, 50% in the p22-OE cells, and only slightly in the p22-KD cells. When the cells were stimulated by AA or serum, the patterns of ROS levels in these three cell lines were similar to that in the PDGF-stimulated cells, except that serum elevated more ROS generation in all three transfectants. In general, the data suggest that p22phox expression level controls NOX activity and ROS generation.

It has been suggested that the amount of ROS is critical in stimulating cell proliferation in many cell types, including lens epithelial cells. Further to understand the consequences of altered expression levels of p22phox in cell proliferation, we measured cell growth by performing a nucleic acid stain–based assay, with a fluorescence-generating dye (PicoGreen; Invitrogen) that can bind to DNA, and the relative fluorescence can be detected quantitatively by a fluorometer. Figure 4A shows the comparison of the growth rates among p22-KD, p22-OE, and Vec cells cultured in MEM containing 20% FBS. Knockdown of p22phox significantly interrupted the ability of the cells to

FIGURE 1. Detection of the p22phox subunit of NADPH oxidase in human lens epithelial cells (HLE B3) and the HLE B3 transfectants with manipulated p22phox expression levels. (A) RT-PCR to detect the p22phox gene expression in HLE-B3 cells. The total PCR products were separated by agarose gel electrophoresis. Arrow: p22phox DNA band of 582 bp. M, DNA marker. (B) Western blot analysis of p22phox and gp91phox in the HLE B3 cells with the specific anti-p22phox antibody. Left lane: a single band at 22 kDa of p22phox in the cell lysate (25 μg protein); right lane: the 22-kDa band in the p22phox immunoprecipitant. (C) Western blot analysis for p22phox and gp91phox in HLE B3 transfectants of p22phox-KD, p22phox-OE, and Vec cells. The same membrane was reprobed with G3PD as the loading control.
proliferate; however, overexpression of p22phox enhanced cell proliferation.

To further examine the effect of p22phox level on cell proliferation, we performed a BrdU-based assay of DNA synthesis. Under nonstimulatory conditions, p22-KD cells displayed little new DNA synthesis in comparison to Vec cells, but the p22-OE cells enhanced DNA synthesis 30% above the level of Vec cells (Fig. 3B). When all three cell lines were each stimulated with PDGF, AA, or 20% FBS, the BrdU assay showed an extensive increase in both Vec control and p22-OE cells, but little or no effect on the p22-KD cells. Cells of p22-OE responded to stimulant much more (40%) than the Vec control cells. In the case of 20% FBS stimulation, the proliferation rate in the p22-OE cells was nearly doubled in comparison with the Vec control (Fig. 3B).

To test the possibility that apoptosis may play a role in the slower growth of the p22-KD cells, we determined the level of apoptosis in each of the three transfectants by using an Annexin V-based apoptosis analysis. H2O2-treated HLE B3 cells (150 µM for 1 hour) were used as a positive control. Under untreated conditions, less than 2% of all three transfectants were apoptotic, whereas 12% to 16% of the cells become apoptotic when treated with H2O2 (data not shown). This phenomenon indicates that an altered expression level of p22phox does not affect the health of HLE B3 cells.

To examine the molecular mechanisms of NOX-regulated mitogenic signaling, we used Western blot analysis to detect the activation of several important signaling components, including ERK1/2, JNK, Akt, and p38. Akt is a key factor that controls cell survival and the antiapoptotic process, whereas both ERK1/2 and JNK are known signaling factors in cell proliferation. The signaling factor p38 is related to the stress response and was used as a negative control. In addition, Western blot analysis with anti-p22phox antibody was conducted to ensure that the appropriate transfectants were used for the experiments.

Figure 5 summarizes the patterns of activated signaling components found in all three transfectants during the 30-minute PDGF stimulation. PDGF-stimulated control cells (Vec) displayed a transient activation of ERK1/2 (as P-ERK1/2), which reached maximum at 10 minutes, declined at 20 minutes, and returned to basal level after 30 minutes. Similar transient activations of JNK and Akt were observed in the PDGF-stimulated Vec control cells (Fig. 5A). However, under the same PDGF-stimulated conditions, overexpression of p22phox extensively enhanced the intensity and duration of the transient activations of P-ERK1/2 and P-Akt. Although the effect of
higher cellular p22phox was less on P-JNK, the intensity of activation was shifted to an earlier peak time in the p22-OE cells than in the control cells. Knockdown of p22phox in the cells almost completely eradicated all signals of P-ERK1/2, P-Akt, and P-p38 with specific antibodies. Cells were stimulated with PDGF (20 ng/mL) for 0, 10, 20, and 30 minutes, and each lysate with 20 μg protein was loaded on gel. G3PD was probed for loading control. (B) Western blot analysis of AA-stimulated p22-KD, p22-OE, and Vec for P-ERK1/2, P-JNK, P-Akt, P-p38 using specific antibodies. Cells were stimulated with AA (60 μM) for 0, 10, 20, and 30 minutes, and each lysate with 20 μg protein was loaded on the gel. G3PD was probed for loading control. (C) Western blot analysis of PDGF-treated p22-KD, p22-OE, and Vec cells for ERK1/2, JNK, Akt, p38, and G3PD using specific antibodies. The conditions of PDGF treatment was the same as described in (A).

Because we observed previously that AA is downstream of the PDGF mitogenic signaling,11 in a separate experiment, we used AA to induce activation of the same signaling components in each of the three cell lines. As shown in Figure 5B, AA induced stimulation patterns of ERK1/2, JNK, and Akt almost identical to that of PDGF in HLE B3 cells (Fig. 5A), except that the P-Akt intensity was lower. AA stimulation showed no effect on P-p38, confirming the specificity of PDGF stimulation in these cells. The similar intensity of G3PD in each lane indicates that equal amounts of proteins were used in gel loading (Figs. 5A, 5B).

The cytosolic p47phox is an important NOX subunit, which can interact directly with p22phox to mediate membrane translocation and binding of other subunits, such as p67phox and p40phox, which are necessary for the full activation of NOX. To investigate the translocation pattern of p47phox to p22phox-comprehended membrane complex induced by PDGF, we isolated membrane fractions from total HLE B3 cell lysates and detected the distribution of p47phox by Western blot analysis. p47phox was located mainly in the cytosolic fraction, with only trace amounts found in the membrane (Fig. 6A). However, p47phox was translocated onto the membrane within 10 minutes of PDGF stimulation and quickly returned to the basal level after 20 minutes, corresponding with the transient activation of signal components for cell proliferation shown in Figure 5A.

We also investigated the potential binding of NOX cytosolic subunits (p47phox, p67phox, and p40phox) to p22phox-comprehended NOX membrane-bound subunits during PDGF stimulation. The experiment was performed by immunoprecipitation of p22phox in the cell lysate from quiescent and PDGF-stimulated (15 minutes) cells, with the specific anti-p22phox antibodies. The p22phox immunoprecipitant was then analyzed for the presence of p47phox, p67phox, and p40phox with specific anti-p47phox, anti-p67phox, and anti-p40phox antibodies, respectively. As depicted in Figure 6B, Western blot analysis showed weak but positive reaction of each cytosolic subunit with its respective antibodies in the p22phox immunoprecipitants of the quiescent cells, and the intensity of each was increased in the post-PDGF-stimulated cells, indicating that immunoprecipitation of p22phox also brought down with it...
the three bound NOX cytosolic subunits of p67phox, p40phox, and p47phox. Equal level of p22phox was found in both the resting and stimulated cells. These results suggest that PDGF stimulates the interactions between the cytosolic subunits and the membrane-bound subunits of NOX.

To further investigate whether the interaction between p22phox and p47phox depends on the level of p22phox expressed in the cells, we examined the p22phox immunoprecipitants from the cell lysates of p22-KD, p22-OE, and Vec transfectants. The immunoprecipitants of each were probed with anti-p47phox antibody. As shown in Figure 7, the interaction between p22phox and p47phox is induced in the post-PDGF-stimulated (15 minutes) Vec and p22-OE cells but not in p22-KD cells. Furthermore, the interaction between p47- and p22phox was enhanced extensively even in the quiescent high p22phox-expressing cells in compared with the Vec control. These results indicate that the cytosolic p47phox most likely forms a binding complex with p22phox at the membrane and the degree of binding seems to depend on the protein level of p22phox in the cells. As p47phox-p22phox binding is the first step in NOX activation, our data also suggest that higher p22phox in cells lead to higher NOX activation.

PDGF binding-induced activation of PDGF receptor requires phosphorylation of Tyr857 residue at the receptor.25 To further understand the role of NOX in the activation of PDGF-triggered signaling, we examined the level of P-Tyr857 in PDGF-treated HLE B3 cells with different expression levels of p22phox. We observed that PDGF (20 ng/mL) transiently enhanced Tyr857 phosphorylation of PDGF receptor in the control cells, and the signal reached maximum at 10 minutes and subsided after 20 minutes of PDGF treatment. Overexpression of p22phox enhanced and extended the level of P-Tyr857 to 30 minutes after PDGF stimulation. However, cells with knocked-down p22phox expression showed little or no response to PDGF stimulation (Fig. 8A), indicating that phosphorylation or activation of Tyr857 at the PDGF receptor is likely regulated by the level of p22phox subunit in the cells. To confirm the enhanced phosphorylation of PDGFR induced by PDGF in p22-OE cells is due to the increased ROS levels, we pretreated the cells with NAC, an antioxidant, and found that P-Tyr857 in the receptor was decreased with increasing concentration of NAC (Fig. 8B). This result confirms that PDGF-induced ROS can control the activity of the active site at the PDGF receptor.

As the oxidation-sensitive LMW-PTP is suggested as one of the target phosphatases for ROS oxidation and inhibition during PDGF mitogenic action,25 we investigated the role of LMW-PTP in PDGF-induced cell signaling in relationship to p22phox of NOX. Because there is no direct activity assay for LMW-PTP, this enzyme was immunoprecipitated first from the cell lysates of p22-KD, p22-OE, or Vec cells and then assayed according to Xing et al.25 LMW-PTP displayed the highest activity in quiescent cells from p22-KD cells, followed by the Vec control cells and was least in p22-OE cells, indicating that p22-KD probably has the least amount of intracellular ROS to inhibit LMW-PTP (Fig. 8C). During the 15-minute PDGF stimulation, LMW-PTP activity decreased in all three transfectants, but the degree of suppression was negligible in the p22-KD cells, moderate (~30%) in the control cells, and severe (60%) in the p22-OE cells. The data suggest that PDGF induced more ROS in cells with higher p22phox expression, thus leading to more inactivation of LMW-PTP.

Finally, Rac1 is another cytosolic subunit for NOX in many other cell types and is thought to interact with NOX complex by binding with gp91phox.25 As we have observed in the past, Rac1 is essential in PDGF-stimulated ROS generation and cell proliferation.12 We examined whether Rac1 can be translocated onto the membrane on either PDGF or AA stimulation in HLE B3 cells. As shown in Figure 9, Rac1, which was found mostly in the cytosolic fraction in quiescent cells, was translocated onto the membrane fraction when the cells were stimulated with PDGF or AA. These results suggest that Rac1 most likely interacts with NOX complex during the mitogenic stimulation induced by PDGF.

**DISCUSSION**

As far as we know, our findings in this study are the first evidence that human lens epithelial cells have an active and
functional NADPH oxidase (NOX) system. The presence of p22phox subunit was confirmed by detecting both its mRNA and protein in the HLE B3 cells. Both p22phox and gp91phox proteins were also detected in the human lens epithelial-cortical region for the first time. This study further demonstrated that primary mouse lens epithelial cells also displayed NOX activity, which responded to PDGF stimulation to produce ROS, similar to that of HLE B3 cells. Furthermore, our recent publication\(^2\) also provides strong evidence that cultured primary mouse lens epithelial cells display the same physiological phenomenon regarding the relationship of PDGF, ROS, and cell signaling, similar to the B3 cells. Therefore, we conclude that the HLE B3 cell is an appropriate model to study NOX function and regulation. In addition, HLE B3 cells are rich in PDGF receptor and fast growing; both make it highly desirable as a model system for such an investigation.

The sequence of the cloned p22phox in HLE B3 cells was found to be identical with that of the human p22phox from other cell types. We also detected gp91phox (NOX2) and p22phox proteins in the human lens tissue and cells, similar to phagocytes and other nonphagocytic cells. All other subunits in the NOX2 system, including p40phox, p47phox, and p67phox and Rac1, were also identified in the HLE B3 cells, confirming the initial findings of Rao et al.\(^1\)

Zhang et al. (\textit{IOVS} 2008;49:ARVO E-Abstract 2279) recently reported that besides NOX-2, the NOX1, -3, -4, and -5 homologues are present in the HLE B3 cells. We speculate that the functional NOX for producing ROS in the HLE B3 cells may be the NOX2 system. This hypothesis is based on our current and previous findings: First, PDGF-stimulated cells could activate NOX to produce ROS when the NOX2-specific cytosolic subunits of p40-, p47-, and p67phox were translocated to the membrane, forming a complex with the membrane-bound p22phox (Figs. 6A, 6B). Second, PDGF-stimulated HLE B3 cells were very sensitive to DPI,\(^1\) which is an inhibitor commonly used to inhibit flavoproteins, including NOX2. Third, PDGF mitogenic action depended on the constitutive presence of Rac1,\(^2\) and that Rac1 was found to translocate onto the membrane in PDGF-stimulated HLE B3 cells (Fig. 9). However, pan-PKC translocation is also needed for ROS production in PDGF-stimulated cells.\(^1\) Whether this PKC process is Ca\(^{2+}\)-dependent and which isoforms of PKC is involved are not clear. Further investigation is needed in this area.

Some previous studies have shown that p22phox could modulate overall NOX activity and thus control various physiological functions in many types of nonphagocytic cells, such as aorta endothelial cells,\(^9\) kidney cells,\(^7\) and vascular smooth muscle cells.\(^25,26,35\) In our present study, the correlation between the expression levels of p22phox and the levels of ROS generation suggests that p22phox participating in the NOX system of HLE B3 cells is crucial. In particular, p22phox could not only control the activity of NOX but also the activation of P-Tyr857 of the PDGF receptor (Fig. 8).

The p22-OE and -KD cells that we established altered only the p22phox expression level without affecting gp91phox; thus, these cell lines may be useful models for examining the importance of p22phox in regulating NOX activity and cellular function. It was suggested that p22phox is a stabilizer for gp91phox, as gp91phox becomes unstable in the absence of p22phox, and patients deficient in p22phox showed no NOX2 protein.\(^3\) Our results, however, indicated that gp91phox protein was quite stable and was maintained at the same level in the cells where p22phox expression was diminished almost
completely (Fig. 1C). This suggests that the interrelationship between p22- and gp91phox in the lens is probably different from that of the phagocytic cells. Further work is needed in this area.

The stable p22-KD cell line may contain some residual p22phox, as a low level of p22phox protein was detected. This incomplete p22phox gene knockdown probably contributed to the residual NOX activity, the weak cell signaling, and the low level of cell proliferation (Figs. 3, 4) observed in the p22-KD cells. Furthermore, NOX5, which was identified in the HLE B3 cells (Zhang W, et al. IOVS 2008;49:ARVO E-Abstract 2279), which is known to be independent of p22phox interaction for activity, may also contribute to some of the residual NOX activity observed in the p22-KD cells. In addition, p22-OE cells showed a strong expression of p22phox, which resulted in higher NOX enzyme activity that correlated with the results of higher PDGF-stimulated ROS generation, stronger growth rate, and intensified redox signaling in the cells. The negative results from apoptosis studies on these cells clearly ruled out the possibility that cell death played any role in the differential growth rates observed between the p22-KD and -OE cell lines.

It is interesting to note that extracellular AA, which is known to stimulate NOX activity along with cell growth and redox signaling by circumventing the receptor, could also respond to the expression level of p22phox, parallel to that of PDGF. Similar to our earlier report, PDGF and AA stimulated MAPK activation in a transient manner and exerted its positive effect on ERK1/2 and JNK, but not on the p58 pathway. However, the transient activation on the survival factor Akt was most pronounced. This finding is to be expected as it has been demonstrated previously that PI3K, the upstream kinase of Akt, is essential in ROS production and PDGF-stimulated cell proliferation, and that inhibition of PI3K could completely eradicate ROS generation and PDGF mitogenic function. Therefore, the redox signaling pathways used for PDGF (and AA) stimulation in HLE B3 are similar to those of other cell types.

We also demonstrated that PDGF stimulation caused the other subunits, including p47-, p67-, and p40phox, to be translocated from cytosol to the membrane and bind with p22phox. The transient binding among these subunits correlated with the activation patterns of ERK1/2, JNK, and Akt. Furthermore, the expression level of p22phox correlated with the binding intensity between p22- and p47phox. Our data agree with the findings of the angiotensin II-induced hypertrophy in vascular smooth muscle in which the NOX activity was potentiated in mice overexpressing p22phox, but completely inhibited in cells transfected with antisense p22phox.

Most interesting, the expression level of p22phox could modulate Tyr857 phosphorylation at the receptor of PDGF. Our data also confirmed that Tyr857 activation was ROS-dependent, as the cells pretreated with NAC diminished Tyr857 activation. We found that Tyr857 phosphorylation was controlled by LMW-PTP; the LMW-PTP activity was transiently inhibited in PDGF-stimulated cells. Therefore, it is likely that some of the ROS produced in activated NOX may be targeting LMW-PTP to allow amplification of the mitogenic signal for cell proliferation. Indeed, when p22phox expression was low in p22-KD cells, the level of P-Tyr857 on PDGFR was negligible, but the LMW-PTP activity was high. In contrast, when p22phox expression was high in p22-OE cells, the level of P-Tyr857 on PDGFR was intensified whereas LMW-PTP was severely inactivated.

An important role of Rac1 in NOX activation has been speculated, both in the phagocytic and nonphagocytic cells. We have also demonstrated in HLE B3 cells that the mutant of constitutively active Rac1 responded to PDGF stimulation by producing more ROS with higher cell proliferation while dominant negative Rac1 showed an opposite effect. Thus, Rac1 is essential in the redox signaling process for PDGF. As Rac1 has been indicated to bind with the membrane subunit gp91phox, we speculate that Rac1 is unlikely to be present in the binding complex of p22phox. Our observation that Rac1 responded to PDGF or AA stimulation by being translocated onto the cell membrane may indicate an interaction between Rac1 and gp91phox. More work is needed in this area.

With these current findings, we expand our earlier hypothesis on the ROS-mediated PDGF mitogenic signaling pathway to include the interrelationship of NOX with LMW-PTP and PDGF receptor. As shown in Figure 10, on PDGF binding, the dimerization and autophosphorylation of the receptor activate ERK pathway, which together with Rac1 activates and translocates cPLA2 to the membrane to allow AA release from the membrane phospholipids. AA therefore can positively feed-back and amplify the signaling cascade by activating NOX to generate ROS. This process is achieved by activating and translocating PKC for the phosphorylation and activation of NOX subunits for p22phox binding. ROS generated from NOX then targets the oxidation-sensitive LMW-PTP whose inactivation allows the activation of Tyr857 on the PDGF receptor. Thus, the cascade of the downstream signaling components from PDGFR can be further amplified to induce appropriate gene expression for various cellular functions.

In summary, we demonstrated that the human lens epithelial cells contain a functional NOX enzyme complex that is similar to other cell types. We also found p22phox to be an important subunit of NOX in regulating not only the enzyme activity and ROS production, but also in the activities of PDGF receptor and its antagonist LMW-PTP for PDGF-induced mitogenic signaling and cell proliferation.

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### References

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