Low Levels of Hydrogen Peroxide Stimulate Corneal Epithelial Cell Adhesion, Migration, and Wound Healing

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Purpose. Intracellular reactive oxygen species have been reported to associate with growth factor and integrin signalings in promoting cell adhesion in many cell types. This study is to explore if exogenous H_2O_2 at low levels can be beneficial to cell adhesion, migration, and wound healing.

Methods. Primary rabbit corneal epithelial cells treated with 0–70 μM H_2O_2 were tested for viability by MTT assay, adhesion by centrifugation assay, focal contacts of vinculin and F-actin by immunofluorescence, activated Src(pY416), EGF receptor (pY845), vinculin(pY1065), FAK(pY397), and FAK(pY576) by immunoblotting. Cell migration was examined with 0–50 μM H_2O_2 using the scratch wound technique. Corneal wound healing of ex vivo pig model and in vivo mouse model was examined using H_2O_2 with and without antioxidant N-acetylcysteine (NAC).

Results. Compared with the untreated control, H_2O_2 at 10–50 μM stimulated cell viability and facilitated adhesion and migration with clear induction of vinculin-rich focal adhesions and F-actin–containing stress fibers by increasing activated Src, FAK(pY576), and vinculin(pY1065). H_2O_2 also increased phosphorylation of EGFR(Y845) parallel to that of activated Src, but both were eliminated by NAC and PP1 (Src inhibitor). Finally, H_2O_2 induced faster wound healing in cornea both in vitro and in vivo, but the healing was diminished by NAC.

Conclusions. These findings suggest that H_2O_2 at low levels promotes cell adhesion, migration, and wound healing in cornea cells or tissue, and the interaction of H_2O_2 with Src plays a major role. (*Invest Ophthalmol Vis Sci.* 2011;52:1723–1734) DOI:10.1167/iovs.10-5866

Reactive oxygen species (ROS) encompass a variety of partially reduced metabolites of oxygen, including superoxide anion ($O_2^-\cdot$), hydroxyl radical ($\cdot OH$), and hydrogen peroxide (H_2O_2). In the past ROS that were generated intracellularly or derived from outside sources were considered to be harmful to cells and tissues. Previous studies have shown that a high ROS level is associated with aging and various degenerative diseases, including macular degeneration and cataracts. In the case of the cornea, damage of the corneal epithelium has been observed due to prolonged use of contact lenses or UVB irradiation[1],[2] In recent years, a growing number of studies have shown that ROS can be generated by a variety of extracellular stimuli such as integrin engagement[3–5] and cytokine or growth factor binding of the receptor in many nonphagocytic cells, including endothelial cells,[6] chondrocytes,[7] epidermoid cells,[8] lens epithelial cells,[9] and corneal epithelial cells.[10] This physiological low ROS level is used to regulate cell signaling and mediate a variety of cellular functions, including cell proliferation, differentiation, and cell adhesion and migration. This newly found ROS function is known as redox signaling, in which ROS act as a second messenger in intracellular signaling pathway by activating protein tyrosine kinases and /or inhibiting protein tyrosine phosphatase and regulating redox-sensitive gene expression.[11],[12] These studies have shed new light on the wide spectrum of biochemical effects elicited by various concentrations of ROS, indicating that maintaining redox homeostasis in cells and tissues is the key to health in general.

More notably, it was recently demonstrated that corneal epithelial cells produced ROS in response to EGF stimulation, and the ROS generated were essential in mediating cell proliferation, adhesion, migration, and wound healing.[10] Chiarugi and colleagues[12,13] elucidated in fibroblast and other cell types that ROS were produced on integrin engagement and ROS were responsible for the direct activation of tyrosine kinase Src by oxidizing its two conserved cysteine (Cys) moieties. The oxidized and activated Src subsequently transphosphorylated epidermal growth factor receptor (EGFR) in a ligand-independent manner, allowing the prosurvival signals to be elicited and escaping from anoikis, or apoptosis due to failed integrin-ECM contact.[14] Src is widely recognized as an early mediator in integrin-triggered signaling and plays a pivotal role in cytoskeletal organization and focal adhesion assembly.[15,16] Activation of EGFR is absolutely required for induction of proliferation and motility in many epithelia, including the corneal epithelium and the epidermis after wounding.[17–19]

Although endogenous ROS has been proved to play an instrumental role in various cell functions, little work has been carried out to determine whether or not extracellular ROS may also provide beneficial effects on cells. A few reports in the literature have addressed H_2O_2-induced cell proliferation and differentiation,[9,20,21] but far fewer in examining the benefit of H_2O_2 for cell adhesion and migration or even wound healing. Understanding whether extracellular ROS can assist such cellular functions, especially in the case of cornea wound healing, is of great ocular therapeutic interest.

Corneal epithelium may be impaired by mechanical or chemical damage or be destroyed by microbial, such as bacterial or fungal, infection. Self-regeneration of the epithelium is essential to maintain corneal transparency and normal vision of the human eye. Typically, the healing takes place within 2 to 3 days after wounding of the corneal epithelium. It starts from

basal cell renewal via cell division and migration of stem cells from the limbus that flatten and form a new cover on the basement membrane. Then a more pronounced proliferation occurs, resulting in an increase in thickness and formation of its normal layers. Corneal epithelial cell adhesion and migration likely rely on the extracellular matrix (ECM)-integrin system and are the most important processes during re-epithelialization and wound healing.[22,23] ECM proteins, such as type IV collagen, laminin, and fibronectin, are components of the corneal basement membrane.[24] When corneal injury results in destruction of the basement membrane, fibronectin is synthesized and deposited at the site of the epithelial defect and serves as a temporary matrix for epithelial cell adhesion and migration.[25,26]

In view of the importance in understanding corneal wound healing, we investigated the probable role of exogenous ROS, namely, H_2O_2, at low concentrations in cell adhesion, migration, and wound healing using cultured primary rabbit corneal epithelial (RCE) cells in the absence of serum growth factor and cytokine, the ex vivo model of pig eye cornea, and the in vivo model of mouse eye cornea. We have found that H_2O_2 at 20 μM in the absence of serum growth factor and cytokine showed no adverse effect on cell viability but actually stimulated adhesion and migration in cultured cells on an Fn-coated plate and enhanced pig eye cornea wound healing ex vivo as well as mouse cornea wound healing in vivo.

MATERIALS AND METHODS

Materials

Supplemented hormonal epithelial medium (SHEM) culture medium consisting of base medium (Ham's F12 and Dulbecco's modified Eagle's medium [1:1], 100 units/mL penicillin, and 100 μg/mL streptomycin), fetal bovine serum (FBS), insulin, dimethyl sulfoxide, trypsin solution, *N*-acetyl-L-cysteine (NAC), MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide], and 30% hydrogen peroxide (H_2O_2) were obtained from Sigma-Aldrich (St. Louis, MO). FBS and EGF were purchased from Gibco Chemical Co. (Grand Island, NY). Neutral protease (Dispase II) was purchased from Roche Molecular Systems (Alameda, CA). PP1, a Src inhibitor, was obtained from Enzo Life Sciences (Farmingdale, NY). Src polyclonal antibody (SRC 2) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for Src(pY416), EGFR(pY845), EGFR, vinculin(pY1065), vinculin, FAK(pY397), FAK(pY576), and FAK were purchased from Cell Signaling Technology (Beverly, MA). Anti-rabbit and anti-mouse immunoglobulin G, horseradish peroxidase–linked secondary antibodies, were obtained from Amersham (Louisville, CO). Other chemicals and reagents were of analytical grade.

Animals

Male New Zealand White rabbits (2.0–2.5 kg) and C57BL/6 mice (25 g) were obtained from Zhejiang Academy of Medical Sciences Laboratory Animals (Zhejiang, China). Fresh pig eyes were obtained from Farmland Meat Packing Company (Crete, NE). Animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Cell Culture

Primary RCE cells were established after the methods described in Huo et al.[10] Briefly, fresh whole corneal lamellar tissue including epithelial layer and superficial stroma was removed from the rabbit eye, treated with neutral protease (Dispase II) to loosen epithelial cells from the basement membrane, and the explants placed on a 100 mm culture dish and covered with SHEM medium and incubated at 37°C in 5% CO_2. The explants were removed when most of the cells had outgrown from the tissues (approximately 4 days), then the cells were

allowed to reach confluence (approximately 7 days). The positive immunohistochemical staining of cytokeratin-3/12 with mouse monoclonal antibodies has confirmed the cells to be corneal epithelial cells.[10] Only the second-to-third-generation passages were used for the experiments.

Primary RCE cells were grown to 80% confluence in the SHEM medium with 5% FBS and 10 ng/mL EGF. Before each experiment, the cells were gradually serum starved (with 0.5% FBS) overnight and then cultured in EGF and serum-free medium for 30 minutes before use.

MTT Assay for Cell Viability

Mitochondrial function, an indicator of cell viability, was measured in RCE cells using the MTT colorimetric assay. Briefly, cells were starved overnight. After that, cells (2×10^5 cells /mL) were incubated with 0, 1, 5, 10, 20, 50, 60, and 70 μM H_2O_2 (diluted with serum and EGF-free medium) for 24 hours, and then medium with H_2O_2 was removed. Cells were then washed 3 times with PBS and incubated with serum- and EGF-free medium containing MTT for 3 hours The tetrazolium salt, which is taken up only by living cells, is metabolized via the action of succinic dehydrogenase to form a colored formazan product, which was extracted by DMSO and quantified colorimetrically at 570 nm in an automated microplate reader (model 550; Bio-Rad, Berkeley, CA).

Detection of Cell Adhesion with Centrifugation Assay

Quantification of cell adhesion was determined after the method of Gao et al.[27] Briefly, cells were starved overnight. After that, cells (70–80% confluence) were detached with 0.25% trypsin (Gibco) for 1 minute, and the trypsin was then blocked with soybean trypsin inhibitor (0.2 mg/mL). The cell suspension was then pretreated with 0, 5, 10, 20, 50, and 60 μM H_2O_2 at 37°C for 15 minutes and plated to a fibronectin (Fn)-coated-96-well plate (BD Falcon; BD Biosciences, Bedford, MA) at 2×10^5 cells /mL. The plates were centrifuged for 3 minutes at 35g at 4°C to let cells attach onto the plate. Then cells were incubated for 30 minutes or 1 hour, after which the plates were inverted and centrifuged. The 30-minute plates were centrifuged at 200g at 4°C for 5 minutes, and the 1 hr plates were centrifuged at 300g at 4°C for 5 minutes to remove the loosely bound cells. The cells remaining on the plates were stained with 0.2% crystal violet (in a 50:50 mixture of ethanol and 0.1 M, pH 4.5 NaH$_2$PO$_4$), and the absorbance at 570 nm was measured by an automated microplate reader.

Cells were serum starved over night and then incubated 2 hours with or without 100 μM NAC before being detached. For comparison, some cells suspension were pretreated with 100 μM NAC alone for 30 minutes, and the cells in another group were pretreated with 100 μM NAC for 15 minutes before exposing to 20 μM H_2O_2 for 20 minutes The centrifugation assay was done using the same process as above after the cells were allowed to adhere to the plate for 60 minutes.

The Effect of H_2O_2 on RCE Cell Attachment to the Extracellular Matrix (ECM): Morphologic Evaluation

Cells were serum starved over night and then incubated 2 hours with or without 100 μM NAC before being detached. Thereby, cell suspensions pretreated without H_2O_2 (0 μM, control group), with 20 μM H_2O_2, with 100 μM NAC alone, and with 100 μM NAC + 20 μM H_2O_2 were each plated onto an Fn-coated 24-well plate. At 15-, 30-, and 60-minute intervals, the suspension was removed from the plate, and cells attached to the plate were fixed in 0.25% paraformaldehyde. Photographs were taken with a phase-contrast microscope (Carl Zeiss, Thornwood, NY).

Immunofluorescence Assay: Actin Cytoskeleton and Focal Adhesion

Cells were serum starved overnight and then incubated 2 hours with/without 100 μM NAC before being detached. Cells suspended (2×10^5

cells/1 mL) and pretreated with either H_2O_2 (0 or 20 μM), 100 μM NAC, or 100 μM NAC + 20 μM H_2O_2 were each plated onto Fn-coated glass slides. After incubation (30 min), cells were fixed with 4% paraformaldehyde for 15 minutes and permeabilized with 0.1% Triton X-100 in 1× PBS for 4 minutes at room temperature. After washing, cells were preincubated for 30 minutes with 1% (w/v) BSA solution in PBS to minimize nonspecific protein-protein interactions. Then cells were incubated with Anti-vinculin (Chemicon International, Billerica, MA) for 1 hour at room temperature. After being washed 3 times with PBS, double-labeling TRITC-conjugated (Chemicon International) phalloidin was incubated simultaneously with the secondary antibody (AP124F; Chemicon International) for 30–60 minutes at room temperature. The staining was observed under confocal fluorescence microscopy (Olympus, Center Valley, PA).

Immunoblotting Analysis: Phosphorylation of Src, EGFR, and Focal Adhesion Molecules on Integrin Engagement

Cells were serum starved over night and then incubated 2 hours with or without 10 μM PP1 or 100 μM NAC before being detached. Thereby, cells were resuspended in serum and EGF-free medium containing with either 0 μM H_2O_2, 20 μM H_2O_2, 100 μM NAC, 100 μM NAC + 20 μM H_2O_2, 10 μM PP1, or 10 μM PP1 + 20 μM H_2O_2 and kept in suspension for 30 minutes and then plated onto an Fn-coated dish for 30 minutes. Cells were then lysed in 50 mM Tris (pH 7.5), 150 mM NaCl, and 1% protease inhibitor (Triton X-100 plus Complete; Roche) and protein tyrosine and serine/threonine phosphatases inhibitors (Millipore, Billerica, MA). Whole-cell lysate, obtained before or after adhesion, containing 15 μg protein was used for immunoblotting analysis. Immunoblotting proteins were eluted by boiling for 5 minutes in a 1× SDS sample buffer containing 5% (v/v) 2-mercaptoethanol. Proteins were resolved (NuPage 4–12% BisTris gel; Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes. The nitrocellulose membranes were immunoblotted with specific antibodies against Src (pY416), Src, EGFR(pY845), EGFR, vinculin(pY1065), vinculin, FAK(pY397), FAK(pY576), or FAK, respectively. Immunoreactive bands were detected using ECL+ regents (Amersham) followed by chemiluminescence on x-ray film.

Scratch Wound Healing Assay In Vitro

RCE cells with 80~90% confluence were serum starved overnight. Thereby, some cells were pretreated in serum and EGF-free medium containing 10 μM PP1 or 100 μM NAC alone for 2 hours, and the cells in other groups were pretreated with 10 μM PP1 or 100 μM NAC for 1 hour before exposing them together with 20 μM H_2O_2 for another hour; other cells were pretreated with 0 (control), 5, 10, 20, or 50 μM H_2O_2 alone for 1 hour. Then cells were wounded by scraping across the cell monolayer with a pipette tip, after the method of Yin et al.[28] After washing with warm PBS, cells were again incubated in the presence of 0 (control), 5, 10, 20, 50 μM H_2O_2, 100 μM NAC, 100 μM NAC + 20 μM H_2O_2, 10 μM PP1, or 10 μM PP1 + 20 μM H_2O_2, respectively. Cell migration or wound closure of the scratched surface was observed and photographed at 0-, 8-, and 16-hour time intervals. After the final phase, the total reoccupied wounded area was quantified with morphometric software (Image-Pro Plus; MediaCybernetics, Bethesda, MD). In total, four different areas were sampled for each group.

Ex Vivo Epithelial Wound Healing in Corneal Organ Culture

The ex vivo wound healing model in organ culture was carried out after the method described in Huo et al.[10] In brief, fresh pig (6–8 months) eyeballs were taken from a slaughterhouse 4 hours postmortem, and the eye globes were trimmed to remove extra tissue and rinsed in PBS containing penicillin (200 units/mL) and streptomycin (200 μg/mL). A blunt scalpel was used to scrape away the epithelium

inside the premarked circle, and then the cornea was surgically excised along with 3–4 mm limbal conjunctiva, rinsed 3 times with PBS, and then mounted on 1% agarose gel and placed in a 60-mm plate. MEM was used to moisten the corneal surface, and the limbal conjunctiva was covered with 5 mL medium. The wounded corneas were allowed to heal for 0, 24, and 48 hours in the medium containing 20 μM H_2O_2 in a humidified 5% CO_2 incubator at 37°C. For comparison, two groups of corneas were pretreated with 40 mM NAC for 30 minutes followed with or without H_2O_2 stimulation. Wounded cornea without treatment was used as the negative control, and unwounded cornea was used for positive control. Four corneas were used for each group. The corneas were stained by Richardson's dye to mark the wounded area at the end of incubation. Before photography (Fotovix; Tamron, Commack, NY), excess dye was removed by rinsing 3 times with PBS. Typically Richardson's dye can provide a well-delineated dark blue stain at the location of an epithelial defect, which is visible under white light and can be photographed without special filters. The wounded areas were quantified with commercial software (ImageProPlus).

Corneal Wound Healing in the Mouse Eye Model In Vivo

C57BL/6 mice (8 weeks, 25g) were anesthetized using intraperitoneal 0.1 mg/g ketamine and topical 0.4% oxybuprocaine hydrochloride (Benoxil; Santen Pharmaceutical Co., Osaka, Japan). A corneal trephine with 2.25 mm diameter was used by gently pressing the central cornea to outline a border of epithelial wound under a dissecting microscope. Then the full thickness of the central corneal epithelium was mechanically removed using a corneal epithelial spatula without damage to the basement membrane. Immediately after scraping, 0.3% ofloxacin ointment (Talivid; Santen Pharmaceutical Co.) was applied to the wounded eyes to prevent bacteria infection. These mice were randomly divided into four groups with four in each, including PBS (control), H_2O_2-treated, H_2O_2 + NAC-treated, and NAC-treated groups. Both eyes in each mouse were instilled 5 μL of the treatment eye drop formula every 6 hours for a duration of 48 hours The eye drop formula contained PBS, 400 μM H_2O_2, 400 μM H_2O_2 + 40 mM NAC, or 40 mM NAC, respectively. Each eye drop solution was made in PBS and adjusted to neutral pH using 5 M NaOH. The size of the corneal wound was monitored under a dissecting microscope after fluorescein staining, and pictures recorded at 0, 12, 24, 36, and 48 hour time intervals. The wounded areas were quantified with commercial software (ImageProPlus). The in vivo experiment was repeated for four different times.

Statistical Analysis

Data were tested for significance by statistical analysis using an unpaired Student's *t* test and one-way ANOVA (SPSS v. 11.0; SPSS, Chicago, IL). All values are expressed as the mean ± SE. Differences were considered significant when $P < 0.05$.

RESULTS

Effect of Low Concentrations of H_2O_2 on the Viability of RCE Cells

To ensure that the concentrations of H_2O_2 to be used for cell adhesion and migration assays are not harmful to the cells, we used MTT assay to examine the effect of 1–70 μM H_2O_2 on the viability of cells. As shown in Figure 1, treatment of RCE cells in Fn-coated 96-well plates with 1–70 μM H_2O_2 in comparison with the untreated control cells (0 μM H_2O_2) showed a dose-dependent relationship between H_2O_2 level and cell viability. The H_2O_2 dose-dependent increase in cell viability was obvious as early as 2 hours posttreatment (data not shown) but was more evident at 24 hours postexposure
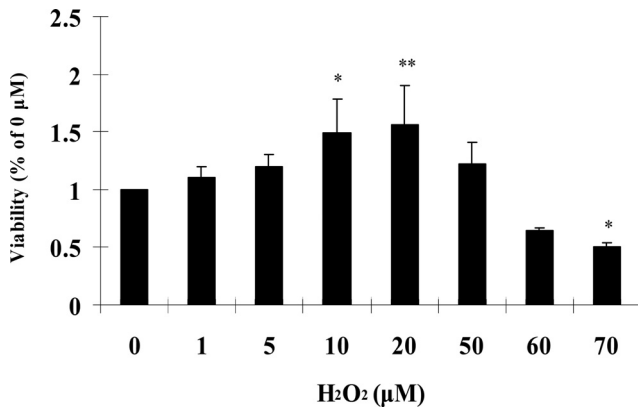
FIGURE 1. Effect of low concentrations of H_2O_2 on the viability of RCE cells. Cultured RCE cells (2×10^5cells /mL) were serum starved (with 0.5% FBS) overnight and then cultured in EGF and serum-free medium for 30 minutes before experiment. Cells were thereby exposed to between (control) and 70 μM H_2O_2 diluted with serum and EGF-free medium for 24 hours. The viability of RCE cells was measured using an MTT assay and data expressed as percentage cell viability compared with control. Results are shown as mean $\pm$ SE ($n = 6$). *$P < 0.05$ and **$P < 0.01$ compared with control.

(Fig. 1), in which 10 and 20 μM H_2O_2 significantly enhanced cell viability to 149.4 $\pm$ 12.1% ($P < 0.05$) and 156.6 $\pm$ 13.8% ($P < 0.001$), respectively, over the control, whereas 60 μM

H_2O_2 reduced cell viability to 65.1 $\pm$ 0.7%, and 70 μM H_2O_2 reduced the viability further to only 50.1 $\pm$ 1.7% ($P < 0.05$) of the control.

Effect of H_2O_2 at Low Concentration on Rabbit Corneal Epithelial Cell Adhesion Examined by Centrifugation Assay

Because H_2O_2 doses <60 μM had no adverse effect on cell viability, we used H_2O_2 doses at the range of 0–60 μM to examine whether low levels of H_2O_2 can enhance cell adhesion. RCE cells of 70–80% confluent were made into suspension and exposed to 0–60 μM H_2O_2 for 20 minutes, followed by adhesion for 30 or 60 minutes before testing cell adhesion using low-speed centrifugation. Apparently H_2O_2 at low levels could significantly enhanced cell adhesion in a dose-dependent manner compared with the untreated control. As shown in Figure 2A, after 30 minutes of post-H_2O_2 treatment, it was found that the 20 μM treatment level increased adhesion 137.8 $\pm$ 7.3%, and the 50 μM treatment level increased adhesion 123.1 $\pm$ 8.7% ($P < 0.05$), respectively, over the control. In the cell population of 60 minute adhesion, 5–50 μM H_2O_2 enhanced cell adhesion in a dose-dependent manner, with a maximum reached 125.7 $\pm$ 2.4% over the control (Fig. 2B). However, when a higher level of H_2O_2 (60 μM) was used, there was a slight adverse effect on cell adhesion (91.3 $\pm$ 11.9%; $P < 0.08$). The data also indicated that H_2O_2-treated cells exhibited a higher increase of adhesion at 30 minutes than that at 60 minutes. Furthermore, as shown in Figure 2C, the
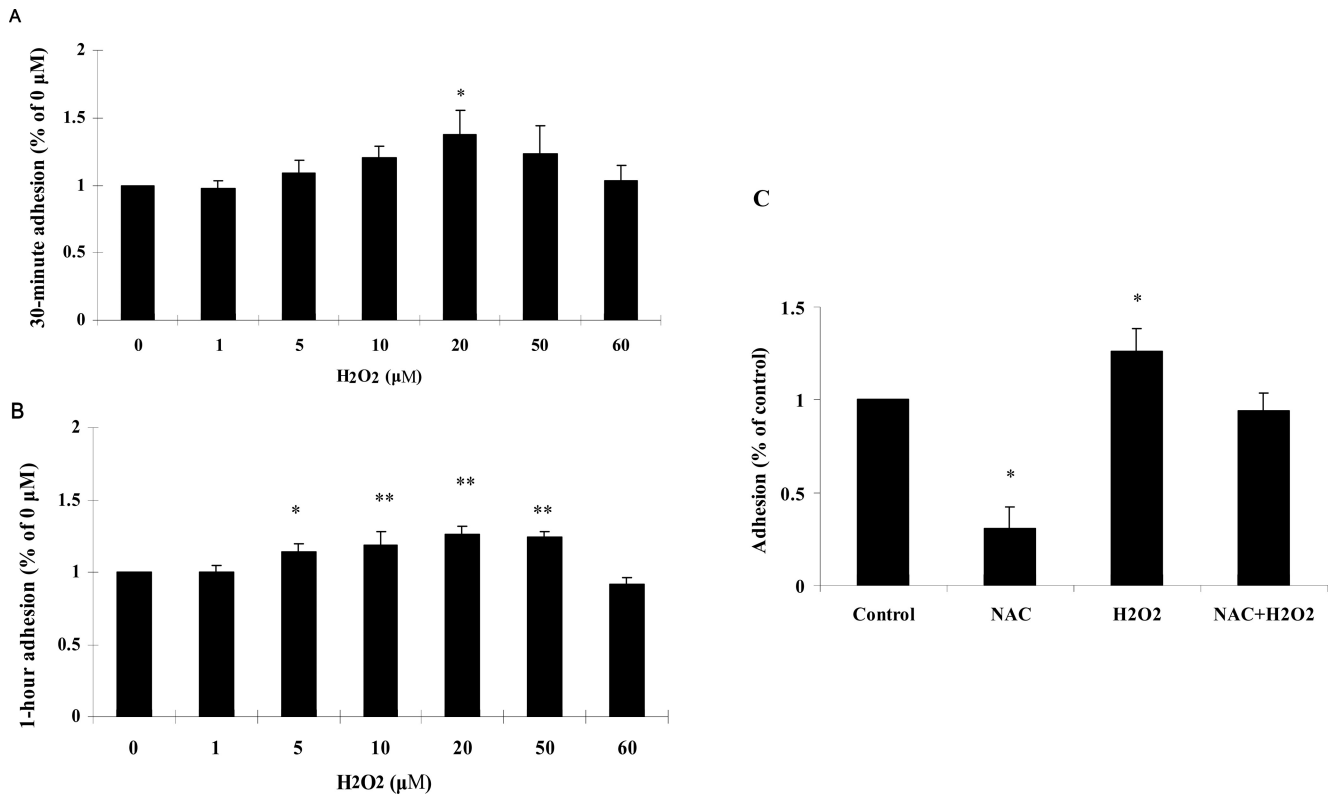

FIGURE 2. Effect of low concentration of H_2O_2 on RCE cell-to-matrix adhesion by centrifugation assay. RCE cells were gradually serum starved as in Figure 1. Cells were then pretreated with between 0 (control) and 60 μM H_2O_2, 100 μM NAC, and 100 μM NAC + 20 μM H_2O_2, respectively, in suspension (2×10^5cells /mL). (**A**) Cells were seeded on Fn-coated dishes in the absence of serum and growth factor with H_2O_2 concentrations of 0, 1, 5, 10, 20, 50, and 60 μM H_2O_2. After 30 minutes, nonadherent cells were removed with centrifugation force at 200g. Adherent cells were fixed and stained with formaldehyde/crystal violet, quantified by measuring the absorbance at OD_{570}. (**B**) Same experiment as (**A**), except that the cells were allowed 60 minutes of adhesion before centrifugation at 300g. (**C**) Comparison of cells treated with NAC alone, NAC + H_2O_2, H_2O_2 alone, and the untreated control in conditions the same as in (**B**). The adhered cells were plotted as the percentage of control (0 μM H_2O_2) and shown as mean $\pm$ SE (*$P < 0.05$ and **$P < 0.01$ vs. control; $n = 6$).

0 μM H$_2$O$_2$ 20 μM H$_2$O$_2$ 100 μM NAC NAC+20 μM H$_2$O$_2$

FIGURE 3. Low-concentration H$_2$O$_2$ accelerates RCE cell attachment to ECM. RCE cells were serum starved and pretreated with 0 μM H$_2$O$_2$, 20 μM H$_2$O$_2$, 100 μM NAC, and 100 μM NAC + 20 μM H$_2$O$_2$ as described in Figure 2. Cells in each group were plated onto an Fn-coated dish for 15 minutes, 30 minutes, and 60 minutes, respectively. Cells were then fixed, and individual fields from multiple experiments were photographed by phase-contrast microscopy. The result is representative of three independent experiments. Arrow indicates cells with polarity shape.
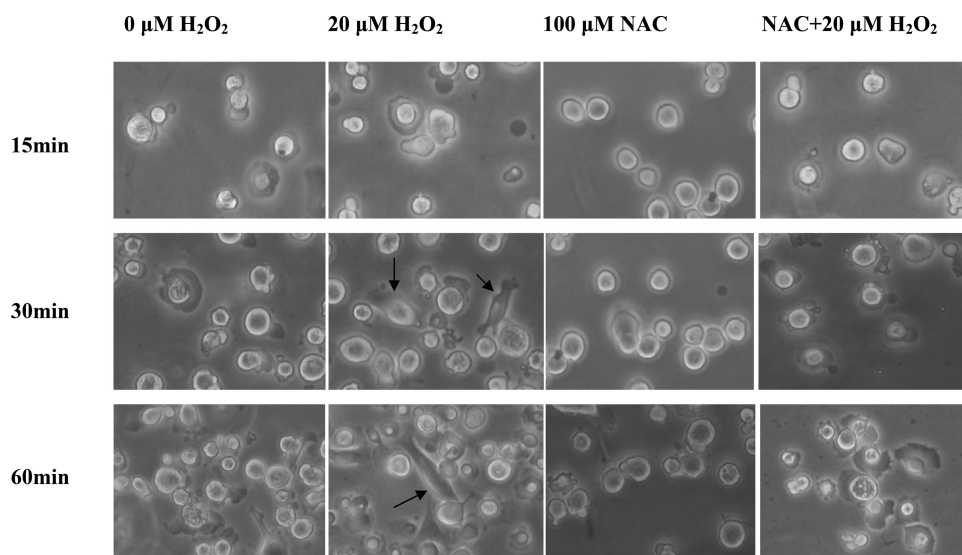
positive effect of 20 μM H$_2$O$_2$ on cell adhesion at 30 minutes posttreatment was eradicated when the cells were cotreated by a general ROS scavenger, NAC (100 μM). Additionally, NAC alone under the same culture conditions suppressed RCE cell adhesion extensively to only $30.6 \pm 3.3\%$ ($P < 0.05$) of the control (Fig. 2C). These data indicate that cell adhesion can be induced by H$_2$O$_2$ at low levels.

Low Concentration of H$_2$O$_2$ Accelerates Corneal Epithelial Cell Attachment to ECM

In a separate experiment, we examined how well H$_2$O$_2$ can stimulate RCE cells to attach to ECM by observing the morphology of cells adhered to an Fn-coated plate under a phase contrast microscope. As shown in Figure 3, H$_2$O$_2$-treated cells (20 μM) exhibited a elongated polarity-shape (arrow) more quickly and progressively than the nontreated cells during the attachment to ECM from 15 to 60 minutes (Fig. 3). In particular, H$_2$O$_2$-treated cells in the 30-minute group showed a more striking difference in acquisition of the cell polarity than those in the 15- and 60-minute groups. This phenomenon, being in line with Fig. 2, indicates that cells spreading at 30 minutes after H$_2$O$_2$ treatment might be the most dramatic phase of H$_2$O$_2$-induced changes. In addition, the presence of NAC (100 μM) antagonized the H$_2$O$_2$–facilitated increase in adhered cells (60 minutes in lower panel of Fig. 3) and that NAC alone showed even less

attachment with lower cell density than that of the control (60 minutes in the lower panel of Fig. 3).

Low Concentration of H$_2$O$_2$ Stimulates Focal Adhesion Formation and Cytoskeleton Rearrangement

We further examined the effect of H$_2$O$_2$ on focal adhesion and cytoskeleton rearrangement by examining the presence of vinculin (a universal maker of focal adhesion) and F-actin during the cell adhesion process using an immunofluorescent detecting technique with anti-vinculin and phalloidin, respectively. Staining of vinculin (green) and F-actin (red) was observed under a confocal fluorescence microscope. Panel 1 in Figure 4 represents a positive control of cells after culturing on an Fn-coated cell plate with normal medium (containing 10 ng/mL EGF and serum) overnight to ensure full adhesion. The well-formed F-actin and vinculin during adhesion served as a visual guide for this study. RCE cells pretreated with 20 μM H$_2$O$_2$ (in serum and EGF-free medium) for 30 minutes displayed a filament-like actin and a dot-like vinculin (panel 3), which were absent in the 0 μM H$_2$O$_2$-treated cells (panel 2). Under the same conditions, NAC (100 μM) antagonized H$_2$O$_2$ (20 μM)–facilitated function in enhancing the formation of vinculin-containing focal adhesion and F-actin–containing stress fibers (panel 5), and that NAC alone reduced the cytoskeleton assembly in the cells even more so than the untreated control (Fig.
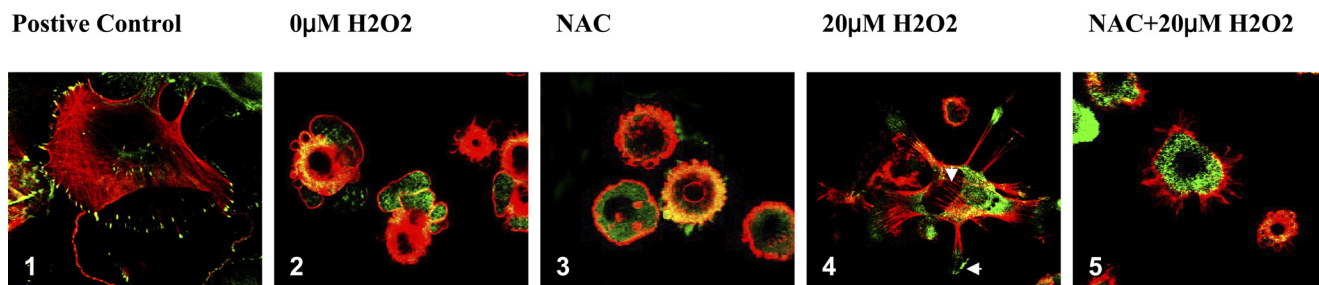
Postive Control **0μM H2O2** **NAC** **20μM H2O2** **NAC+20μM H2O2**

FIGURE 4. The effect of low concentration of H$_2$O$_2$ on focal adhesion and stress fiber formation. RCE cells, which grew to confluence in normal medium overnight, were taken as the positive control (panel 1). Cells pretreated with either 0 μM H$_2$O$_2$, 20 μM H$_2$O$_2$, 100 μM NAC, or 100 μM NAC + 20 μM H$_2$O$_2$ were plated onto an Fn-coated dish for 30 minutes, fixed, and stained for F-actin (*red*) and vinculin (*green*). Cells pretreated with 20 μM H$_2$O$_2$ (panel 4) revealed filament-like actin (*arrow*) and dotlike focal adhesions containing vinculin (*arrowhead*), which were not present in the 0 μM H$_2$O$_2$-treated cells (panel 2). NAC (100 μM) antagonized 20 μM H$_2$O$_2$–facilitated formation of the focal adhesion of vinculin and F-actin (panel 5), whereas NAC alone (panel 3) showed the same results as the cells without H$_2$O$_2$ treatment in panel 2.

4A, panel 4). These data suggest a central role of H_2O_2 in cell adhesion to ECM proteins and cytoskeleton rearrangement.

H_2O_2 at Low Concentration Phosphorylates Src and EGF Receptor on Integrin Engagement

It was reported that Src activation associated closely with integrin engagement, triggered by the released ROS on cell-ECM contact, and that antioxidant could suppress Src activation and attenuate focal adhesion formation during cell spreading.[3,13] To verify if H_2O_2 could also induce Src activation and its downstream processes, we first analyzed the levels of activated Src(pY416) in cells after adhesion in the presence and absence of 20 μM H_2O_2. To test this, Western blot analysis was performed to detect the activated or phosphorylated Src(Y416) and total Src by using specific antibodies against Src(pY416) and Src, respectively. As shown in Figures 5A and 5B, the basal level of Src(pY416)/Src was increased to 136.2 $\pm$ 3.3% ($P <$

0.05, $n = 3$) over the control when H_2O_2 was present. In another experiment, NAC (100 μM)-treated cells almost completely abolished Src phosphorylation at Y416 upon cell adhesion, although cotreatment of NAC with H_2O_2 restored activated Src to its basal level. Next, we test if inhibiting Src with a specific inhibitor (PP1) would affect the H_2O_2-induced oxidation and activation on Src. As indicated in Figures 5A and 5B, PP1 (10 μM) completely eradicates all Src(pY416) in cells regardless of the presence of H_2O_2.

Because it is known that the redox-controlled Src also regulates the phosphorylation of EGFR tyrosine at 845 in several environmental settings, including integrin-mediated cell adhesion and migration,[14,18,19] we next explored if H_2O_2 at 20 μM might phosphorylate EGFR(Y845) in accordance with the elevated Src(pY416). We used specific antibodies to detect the expression levels of EGFR (pY845) and total EGFR in the same immunoblot described above. As shown in Figures 5C–D, cells
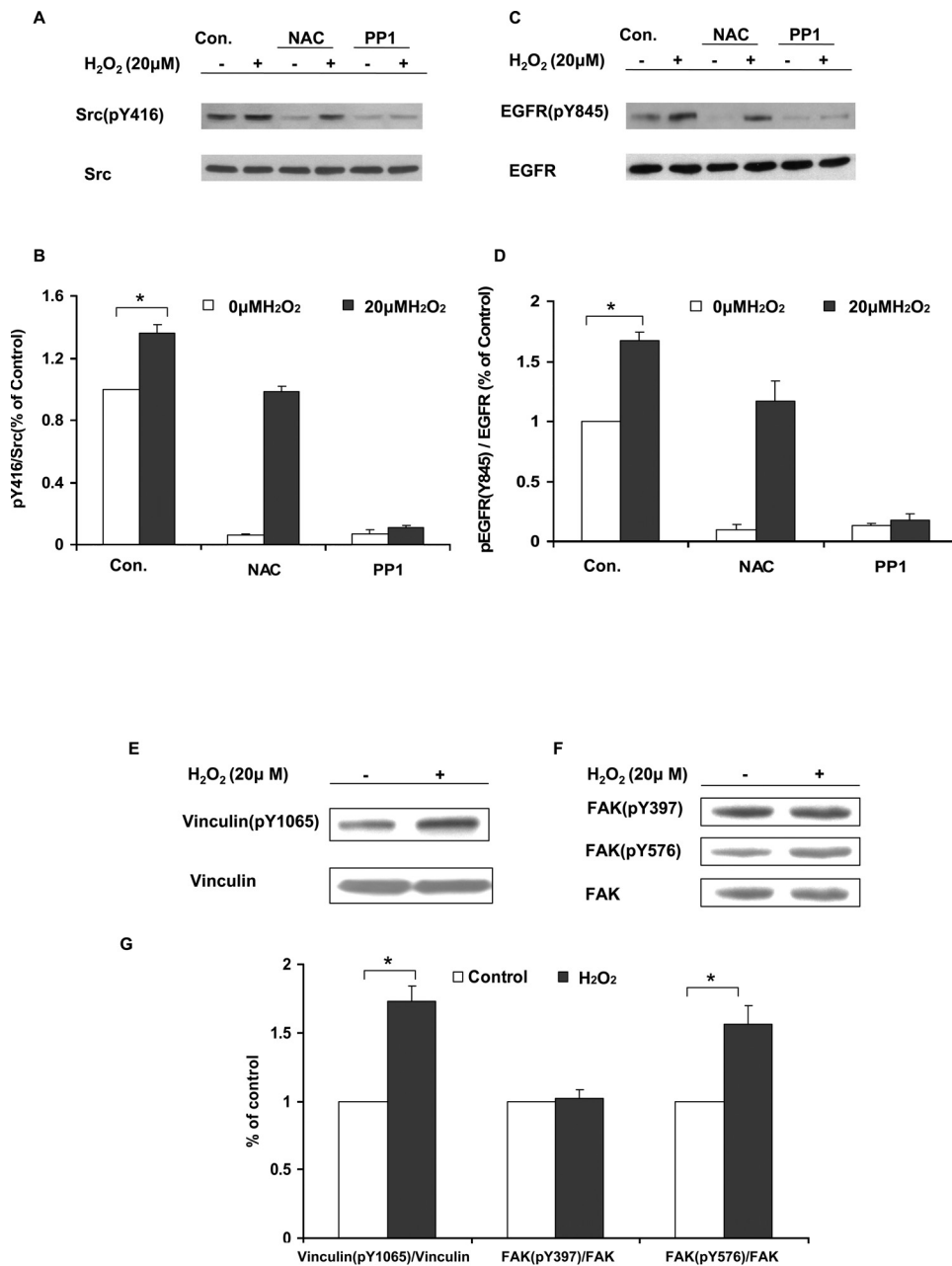

FIGURE 5. Low concentrations of H_2O_2 increases Src activity on integrin engagement. (**A**) Cells were resuspended in serum and EGF-free medium containing either 0 μM H_2O_2, 20 μM H_2O_2, 100 μM NAC, 100 μM NAC + 20 μM H_2O_2, 10 μM PP1, or 10 μM PP1 + 20 μM H_2O_2 and kept in suspension for 30 minutes. Thereby, cells were plated onto an Fn-coated dish for 30 minutes, then lysed and immunoblotted with antibodies against Src(pY419) and total Src. (**B**) The ratio of Src(pY419)/Src was quantified by densitometric scanning of immunoblots and plotted as a percentage of control (0 μM H_2O_2) and shown as mean $\pm$ SE (*P < 0.05 vs. control; $n = 3$). (**C**) Cells were processed as in (**A**) and then lysed and immunoblotted with antibodies against EGFR(pY 845) and EGFR. (**D**) The ratio of EGFR(pY 845)/EGFR was quantified as in (**B**). (**E**) Cells pretreated with 0 μM H_2O_2 or 20 μM H_2O_2 were plated onto an Fn-coated dish for 30 minutes, lysed, and immunoblotted with antibodies against vinculin(pY1065) and total vinculin, as well as (**F**) FAK(pY397), FAK(pY576), and FAK. (**G**) The ratios of vinculin(pY 1065)/vinculin, FAK(pY 397)/FAK, and FAK(pY 576)/FAK were quantified by densitometric scanning of immunoblots and plotted as percentage of control (0 μM H_2O_2) and shown as mean $\pm$ SE (*P < 0.05 vs. control; $n = 3$). Con., control.

treated with H_2O_2 increase the ratio of EGFR(pY845)/EGFR by $67.5 \pm 7.1\%$ ($P < 0.01$) over the untreated control (basal level). Parallel to the results of Src in Figure 5A, NAC inhibited EGFR (pY845), and the inhibition could be partially reversed by H_2O_2, but the suppressed EGFR (pY845) by PP1 could not be rescued by H_2O_2. These data suggest that EGFR (pY845) is Src dependent and oxidation dependent. The above immunoblot analysis showed an equal level of Src or EGFR, indicating that an equal amount of proteins were loaded on the gel.

Finally, because focal adhesion assembly molecules such as FAK(Y576) and vinculin(Y1065) are known to be Src dependent for phosphorylation,[15,29] we conducted another immunoblot analysis to compare the expression status of vinculin(pY1065) (Fig. 5E) as well as FAK(pY397) and FAK(pY576) (Fig. 5F) in cells with and without H_2O_2 treatment. As expected, H_2O_2 at 20 μM also increased vinculin(pY1065) and FAK(pY576) at $173.2 \pm 11.1\%$ and $156.4 \pm 14.2\%$ ($P < 0.05$), respectively, over the untreated control (Fig. 5G). However, the same level of H_2O_2 did not affect the integrin-mediated autophosphorylation of FAK at Tyr 397 (Figs. 5F, 5G). Equal amount of vinculin and FAK on the

immunoblot indicates that the same amount of proteins was loaded onto the gel.

Effect of Low H_2O_2 Concentrations on Corneal Epithelial Cell Wound Closure In Vitro

The confluent monolayer of RCE cells grown in an Fn-coated plate was scraped across with a pipette tip to create a long, narrow wounded region without cells. The wound closure or cell migration into the wounding area was examined in 8 and 16 hours, respectively, after incubation in medium containing 0 (control), 5, 10, 20, 50 μM H_2O_2, 100 μM NAC alone, or 100 μM NAC + 20 μM H_2O_2. Figure 6A depicts the progressive reoccupation of the wounded region in 0 time (left panels), 8 hours (middle panels), and 16 hours (right panels) under various supplemented culture medium. In general, the H_2O_2-enhanced wound closure in comparison with the control (0 μM) was concentration sensitive. The most effective H_2O_2 level was 20 μM, in which a partial wound closure in 8 hours and a complete closure in 16 hours were observed. Concentrations either lower (5 or 10 μM) or higher (50 μM) than 20 μM were less effective but
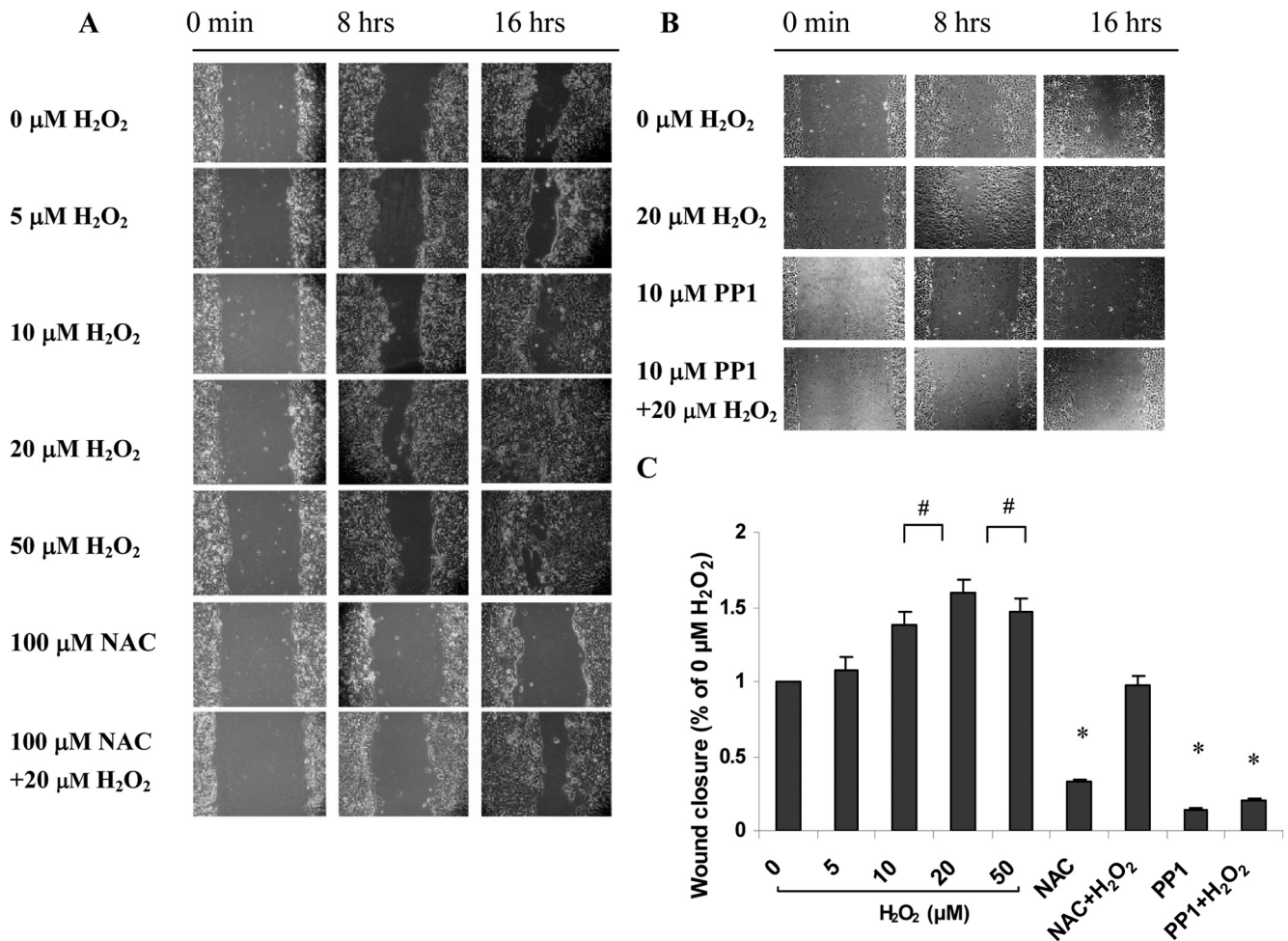

Figure 6. Effect of low H_2O_2 on scratch wound closure in vitro. (**A**) Cells pretreated with 0 (control), 5, 10, 20, 50 μM H_2O_2, 100 μM NAC, or 100 μM NAC + 20 μM H_2O_2 were grown to confluence on Fn-coated 12-well plates, and a scratch wound on the monolayer was created by a sterile plastic pipette tip. Cells were then allowed to migrate into the wound area in the absence of serum and growth factor. Micrographs of cell migration into the scratch-wound region were recorded at 0, 8, and 16 hours under phase-contrast microscope. (**B**) Confluent monolayer of cells pretreated with 0 (control), 20 μM H_2O_2, 10 μM PP1 (Src inhibitor), and 10 μM PP1 + 20 μM H_2O_2 were each made a scratch wound as in (**A**), and the wound closure (migration) was observed and recorded over 0, 8, and 16 hours (**C**). Images of wound closure were analyzed quantitatively at 16 hours. In total, four different areas were sampled for each group. Data are expressed as percentage rate of wound closure in comparison with that of the control and shown as mean $\pm$ SE (*$P < 0.01$, #$P < 0.05$, $n = 3$).

still better than the untreated control. Co-incubation of NAC (100 μM) partially eradicated the beneficial effect of 20 μM H$_2$O$_2$ but better than cells treated with NAC alone. Cells incubated in NAC alone displayed almost no cell migration or wound closure at the end of 16 hours. Quantitative image analysis of the wound closure in 16 hours is shown in Figure 6C, in which 10, 20, and 50 μM H$_2$O$_2$ each significantly accelerate wound closure at 138.4 $\pm$ 8.0%, 159.1 $\pm$ 9.3% and 146.5 $\pm$ 9.1%, respectively, over the control. H$_2$O$_2$ at 20 μM was significantly more effective than the other concentrations ($P < 0.01$). NAC completely prevented H$_2$O$_2$-induced cell migration, and NAC alone suppressed the basal level of cell migration down to 33.5 $\pm$ 1.1% of the control ($P < 0.01$).

It is well known that cell adhesion, spreading, and migration use common signaling pathways. Since Figure 5A showed that low exogenous H$_2$O$_2$ increased the expression of activated Src during cell spreading, we next tested if Src is involved in cell migration or wound healing by using PP1 (a specific Src inhibitor) in the same cell-scraping model used in Figure 6A. The progress of scratch wound closure was monitored and compared among the following groups: 0 (control) μM H$_2$O$_2$, 20 μM H$_2$O$_2$, 10 μM PP1, or 10 μM PP1 + 20 μM H$_2$O$_2$. As shown in Figures 6B and 6C, PP1 alone inhibited the closure of a scratch wound, which is consistent with the published results.[19,30] Moreover, PP1 also blocked the beneficial effect of 20 μM H$_2$O$_2$ on the epithelial wound closure (bottom panels in Fig. 6B), indicating that 20 μM H$_2$O$_2$ facilitates wound closure via the Src pathway. Quantitative image analysis of the wound closure in 16 hours revealed that PP1 alone and PP1 + 20 μM H$_2$O$_2$ slowed the wound closure down to 14.1 $\pm$ 1.2% and 20.4 $\pm$ 1.1% of the control ($P < 0.01$), respectively.

Effect of H$_2$O$_2$ on Wound Healing in Pig Cornea Ex Vivo Model

To prove that H$_2$O$_2$ is beneficial to corneal wound healing, we used a pig cornea organ culture model using the methods of Huo et al.[10] modified according to Xu et al.[31] The wounded corneas were divided into three groups: one received no treatment (control), one treated with H$_2$O$_2$ (20 μM), and the third group pretreated with NAC (40 mM) and then incubated in 20 μM H$_2$O$_2$, respectively. As shown in Figure 6, the excised pig cornea is seen mounted on silica gel with the central stain from the medium. The cornea that received no treatment showed a slight change in the size of the central stain (8.3 $\pm$ 1.2% wound closure) after 24 hours compared with the 0-hour wound, and 71.5 $\pm$ 1.5% progressive wound healing was seen after 48 hours. The cornea that received H$_2$O$_2$ clearly showed a significantly shrinkage of the wounded area (32.4 $\pm$ 1.5%, $P < 0.01$, compared with control) within 24 hours, and a complete healing was seen after 48 hours (97.8 $\pm$ 1.0%, compared with control). In addition, NAC inhibited the beneficial effect of 20 μM H$_2$O$_2$ on the wound closure during the observing time.

Effect of H$_2$O$_2$ on Mouse Corneal Wound Healing In Vivo

To examine whether treatment with eye drops containing a low level of hydrogen peroxide is beneficial to corneal epithelial wound healing in vivo, we used C57BL/6 mice and divided them into four groups with four mice in each. Both corneas of each mouse were scraped at the center area with a spatula to form an epithelial wound. In the first three groups, the mouse eyes were treated every 6 hours with eye drop formulation containing 400 μM H$_2$O$_2$, 400 μM H$_2$O$_2$ + 40 mM NAC, or 40 mM NAC alone. The corneas in the fourth group (control) were treated with PBS without any additive. As shown in

Figures 7A and 7B, eye drops containing 400 μM H$_2$O$_2$ accelerated corneal epithelial wound healing (decrease in green stain) compared with PBS control during the observing time. Wound closure in H$_2$O$_2$-treated corneas completed at approximately 36 hours, whereas wound closure in the PBS-treated corneas had 20.7 $\pm$ 2.6% unhealed area ($P < 0.01$) at the same period and was completely healed around 48 hours (Figs. 7A, 7B). This H$_2$O$_2$ function was inhibited by the presence of NAC. In the group whose corneas were treated by eye drops containing both H$_2$O$_2$ and NAC, the wound was not healed in 36 hours (22.4 $\pm$ 1.9% wound area remained) or at the end of 48 hours (6.0 $\pm$ 3.4% wound area remained). On the other hand, corneas treated with NAC alone showed very slow wound closure, with 38.6 $\pm$ 4.0% and 22.6 $\pm$ 2.5% wound area remaining in 36 and 48 hours, respectively, in comparison with that of the control and H$_2$O$_2$-treated groups. NAC-treated corneas were completely healed only after 72 hours (data not shown). All eye drop formulations used (400 μM H$_2$O$_2$ or 40 mM NAC) did not induce any ocular surface damage either inside or outside the wounded area with no observation of superficial punctuate keratopathy (SPK) or conjunctival congestion.

DISCUSSION

The present study demonstrated that exogenous H$_2$O$_2$ at low concentration has a physiological function in mediating cell adhesion, migration, and wound healing in cornea, parallel to that of epidermal growth factor.[10] Our conclusions are based on the following observations: (1) H$_2$O$_2$ below 50 μM did not adversely affect cell viability; (2) H$_2$O$_2$ in the range of 5–50 μM enhanced cell adhesion by facilitating the formation of focal adhesion complexes and stress fiber; (3) exogenous H$_2$O$_2$ enhances Src activation, resulting in trans-phosphorylated EGFR on integrin engagement; (4) exogenous H$_2$O$_2$ in the range of 10–20 μM promoted cell migration/wound closure in cultured corneal epithelial cells; (5) H$_2$O$_2$ at 20 μM provided a faster wound healing in the pig corneal ex vivo model; and (6) H$_2$O$_2$ (400 μM) formulated in the eye drop stimulated wound healing in mouse cornea in vivo. Although we did not measure the H$_2$O$_2$ level at the end of each experiment, it was evident that when corneal cells or tissues were pretreated with NAC followed by cotreatment of NAC with the effective dosage of H$_2$O$_2$, the beneficial effect was totally prohibited or eradicated. The efficacious dosage is limited to a narrow range of 10–20 μM, indicating that this range may be the physiological concentration of ROS in cells needed for facilitating the redox signaling event, while a higher dosage (> 50 μM) alters the redox balance and leads to oxidative stress to the cells or tissues.

It is well known that after integrin engagement, FAK is autophosphorylated on Y397, which binds to the SH2 domain of Src, and the activated Src kinase in the newly formed FAK-Src complex can phosphorylate focal adhesion components, such as FAK(Y576), vinculin(Y1065), and paxillin, etc., leading to various cellular processes.[15,29] Notably, integrin-elicited ROS are mainly produced during late adhesion to ECM, in strict concomitance with Src activation, and have been suggested to play a major role in focal adhesion formation and cytoskeleton rearrangement.[4,12,32,33] In accordance with these findings, our results (Figs. 5A, 5C) showed that exogenous H$_2$O$_2$ enhanced the supply of activated Src (Src[pY416]) and the subsequent downstream FAK(pY576) and vinculin(pY1065). However, H$_2$O$_2$ had no effect on the integrin-induced autophosphorylation of FAK(pY397), indicating the action of H$_2$O$_2$ is target specific. Our studies also demonstrated that activation of Src was essential for the
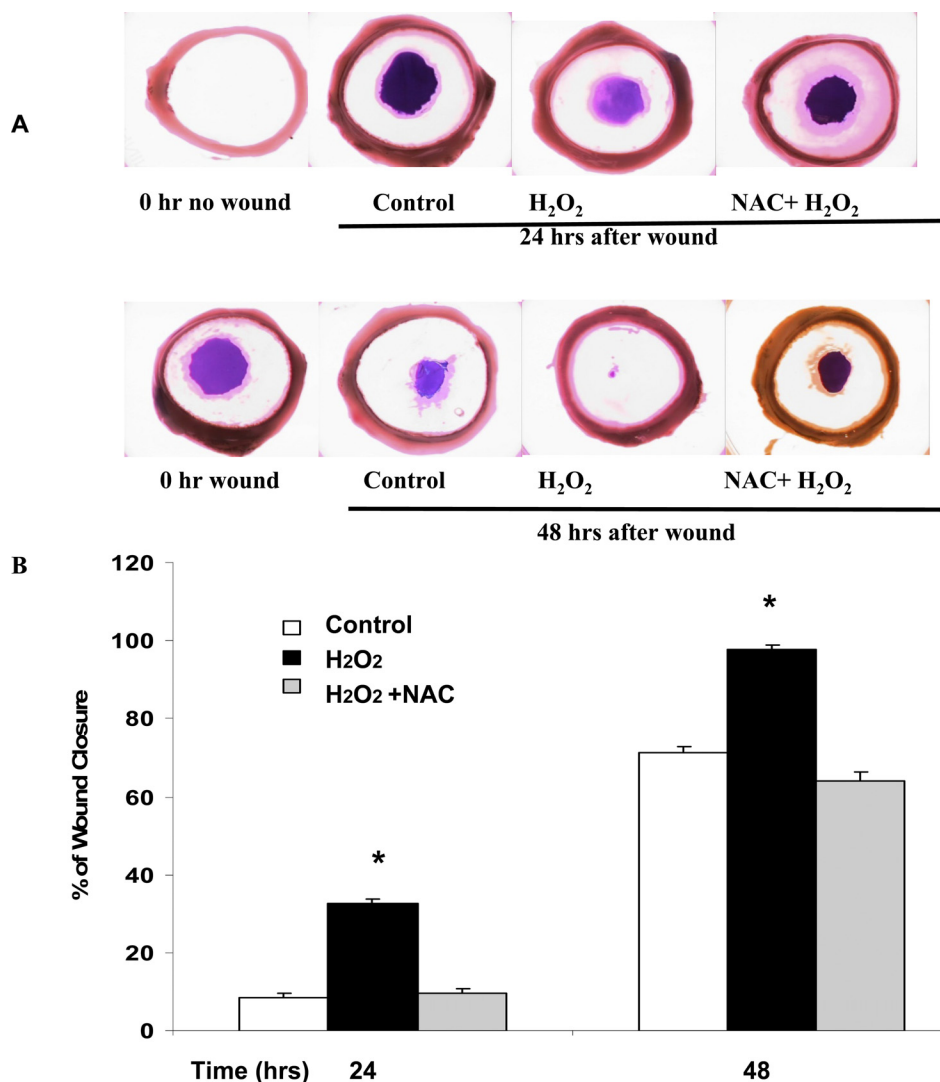
FIGURE 7. The effect of low level of H_2O_2 on wound healing in cornea organ culture. (**A**) The cornea wound on fresh pig eyeball was made using a hollow plastic column (9 mm in diameter) in the center of the cornea. Epithelium inside the circle was removed. The wounded corneas were allowed to heal in the medium containing 20 μM H_2O_2 with or without 40 mM NAC in a humidified CO_2 incubator. After 24 or 48 hours of incubation, the corneas were stained with Richardson's dye for taking photos. A wounded cornea without treatment was used as a negative control. In addition one cornea without wounding and another cornea with wound were stained with Richardson's dye at 0 hour, and the photos were taken and included for comparison. (**B**) Graph represents the percentage of the wound area healed in 24 and 48 hours, shown as mean $\pm$ SE. The data at 24 and 48 hours in H_2O_2 and H_2O_2 + NAC group were compared with the control group (*$P < 0.01$, $n = 3$).

intergrin-dependent EGFR activation, because inhibition of Src (PP1) completely eliminated the expressions of both Src(pY416) and EGFR(pY845) (Fig. 5B). These results corroborate with the findings of Block et al.[18] and Xu et al.[19]

Our data also suggest that ROS is necessary for Src activity on integrin-ECM engagement in RCE cells, since the adhesion-induced Src(pY416) or the basal-activated Src was nearly wiped out by NAC but was restored fully after H_2O_2 treatment (Fig. 5A). These data were consistent with the molecular mechanism elucidated in fibroblast and other cell types that ROS can directly activate tyrosine kinase Src by oxidizing its two conserved Cys moieties during cell spreading.[12,13] Most interestingly, activation of EGFR upon integrin-ECM engagement appeared to be also ROS dependent, similar to that of Src (Fig. 5C). These results confirm the findings and suggestions of Chiarugi and colleagues[3,13] that ROS play an important role in cell adhesion, spreading, and related processes.

Giving the importance of redox-controlled Src in cell adhesion, spreading, and survival as observed by Chiarugi and colleagues,[3,13] and our confirmation of the H_2O_2-induced Src activation, it is reasonable to expect that H_2O_2 could benefit the wound-healing processes as observed in the cell scratch wound model (Fig. 6) and the corneal tissue models in vitro (Fig. 7) and in vivo (Fig. 8). Xu et al.[19] provided evidence that Src was essential in wound healing because inhibiting Src with a specific inhibitor (PP2) could blot the wound healing in the

scratch wound model using corneal epithelial cells. We confirmed their findings by demonstrating in the same model using primary RCE cells. Furthermore, we observed that the retarded cell migration caused by PP1 (an analog of PP2) was unable to be lifted by H_2O_2 (Figs. 6B, 6C). It is likely that PP1 interaction with Src may have caused conformational changes so much so that the two critical Cys become inaccessible to ROS.

Our present study was conducted in the absence of serum or growth factors but showed pivotal phosphorylation of EGFR(Y845) that is known to link with several signaling pathways induced by EGF for cell survival and mitogenic signaling, such as PI-3K and MAPK.[34,35] Thereby, this led us to speculate that a low level of H_2O_2 might mimic growth factor to achieve the above positive physiological events by increasing Src activity. How H_2O_2 activates Src and other elements in cell adhesion, migration, and wound healing and what pathway it takes to achieve some of the observed functions will be the subjects of future studies.

Of all the ROS species, H_2O_2 is the most stable oxidant, and it can diffuse across cellular membranes through water channels and causes oxidative protein modifications at distal areas from its production site or entry point[22]. Therefore, H_2O_2 is often selected as a model agent for oxidation studies. Previously, extensive reports can be found on oxidative injury to cells or tissues induced by a high dose of H_2O_2. In recent years, many new findings suggest that ROS are essential mediators for
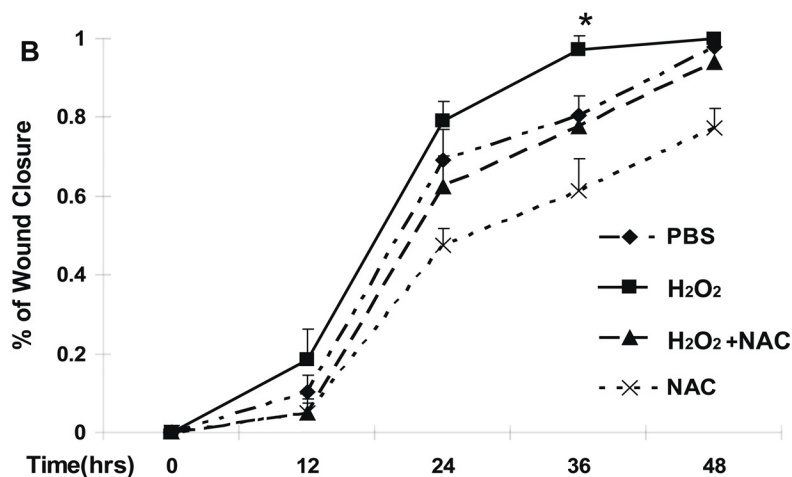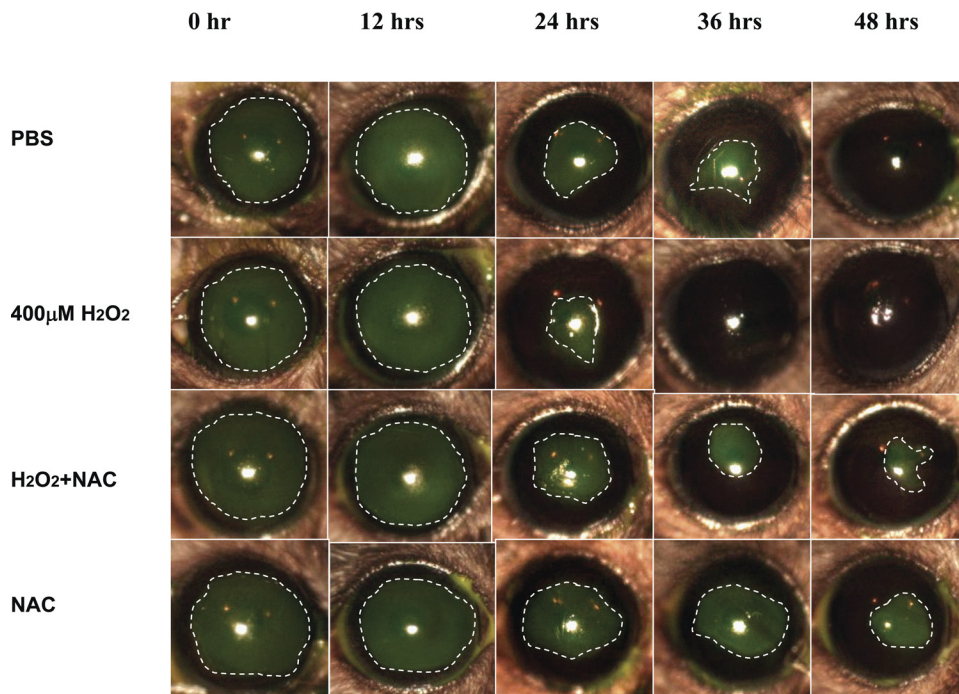
A

B

FIGURE 8. Effect of low H_2O_2 on mouse corneal epithelial wound healing in vivo. (**A**) C57BL/6 mice were used for this in vivo study. Each cornea was scraped at the center area with a spatula to form an epithelial wound. These animals were divided into four groups, and the eyes were treated every 6 hours up to 48 hours with the following eye drop formulations: The first group was treated with an eye drop formulation containing 400 μM H_2O_2; the second group was treated with an eye drop containing 400 μM H_2O_2 + 40 mM NAC; the third group was treated with an eye drop containing 40 mM NAC; and the fourth group (control) was treated with PBS without any additive. Photos were taken at time intervals of 0, 12, 24, 36, and 48 hours after using fluorescein staining of the corneas to allow clear demarcation of the wounding surface (*green*). *White dotted outline* indicates the fluorescein-stained wound region. (**B**) Quantification of the wound healing in the in vivo mouse study. The area of the healed region in each corneal wound was quantified, expressed as percentage rate of wound healing as a function of time (hours) and shown as mean ± SE. The data at 36 hours in H_2O_2, NAC, and H_2O_2 + NAC group was each compared with control group (*$P <$ 0.01, n = 4).

the mitogenic action of growth factor/cytokine in cell signaling. This regulatory function is called redox signaling. Thus, many reports on the positive effect of ROS at low concentration on cells are emerging. Peden et al.[20] noted that low amounts (5–20 μM) of H_2O_2 had no effect on mast cell function, whereas cell proliferation was inhibited by 75–200 μM H_2O_2. Chen et al.[9] reported that 10–20 μM of H_2O_2 could increase cell proliferation in human lens epithelial cells, but concentration higher than 50 μM was harmful. However, Alexandrova et al.[36] reported that 50 μM H_2O_2 could increase Ras activity and enhance cell adhesion and cell motility in rat fibroblast cells. Haendeler et al.[37] also indicated that 10–50 μM H_2O_2 was protective for endothelial cells, whereas 100 μM H_2O_2 exerted cytotoxicity. Our current findings are in line with these reports and confirm that ROS can produce a two-sided effect on cells, depending on the dosage used and the cell type applied.

We used three models to examine the beneficial effect of H_2O_2 at low dosage to cell migration-wound healing, including cell culture, ex vivo, and in vivo models. In each case, we used an effective antioxidant NAC to prove that during cotreatment of NAC and H_2O_2, corneal cells or tissues could no longer heal properly, indicating the beneficial factor is indeed H_2O_2. It is important to point out that a high level of NAC has been considered toxic to cornea in several reports.[10,38,39] Some of the studies used very a high level of NAC (20% or 1.34 M) for treatment. Although no suggestion was made as to the nature of its toxicity, based on our present study, we speculate that the toxic nature of NAC at a high level is over-antioxidation. Because cells require a redox-balanced environment to maintain a healthy status, the treatment of the cornea with a high level of NAC (which strong antioxidation capability can remove the basal level of ROS in cells needed for maintaining general health) can result in a state of redox imbalance with a hypo-oxidative condition. Furthermore, it is clear from Figures 5 and 7 that when NAC was administered alone, the wound healing was extensively retarded, but when NAC was applied together with H_2O_2, the healing process was greatly improved.

Furthermore, the centrifugation assay (Fig. 2C) indicates that NAC alone suppressed cell adhesion extensively, but that suppression was considerably reduced when H$_2$O$_2$ was also present with NAC.

It is worth noting that the antioxidant NAC could be beneficial to cornea wound healing in certain conditions, such as the case of partial alleviation of high glucose-induced retardation in wound healing in cultured human corneal epithelial cells.[40] This opposite effect of NAC in wound healing is reasonable because a high glucose or diabetic condition is known to induce oxidative stress in the cells or tissues,[41] and thus a suitable amount of NAC (25 μM or 25% of the dosage used in our present study) used in the study of Xu et al.[40] may have eliminated part or all of the oxidants derived from hyperglycemia and thereby under those conditions have a beneficial effect on corneal wound healing.

It is difficult to estimate the amount of H$_2$O$_2$ that can be effective in vivo from the amount of H$_2$O$_2$ that has been shown to be effective in vitro. Based on the literature,[42] an estimated efficacious dosage of EGF in vivo is approximately 20-fold higher than that of EGF in vitro (cell culture). It is reasonable to use such an estimation because when using eye drops with any drug formulation for in vivo studies, frequent eye blinking and tear washing can dilute and decrease the actual amount of drug retained and absorbed by the corneal epithelial cells. We applied the same principle to our in vivo studies and used 400 μM H$_2$O$_2$ (20 times the efficacious 20 μM H$_2$O$_2$ in cell culture studies). Despite the fact that H$_2$O$_2$ is a smaller and easily diffusible molecule than EGF, our result in Figure 7 indicates that such approach is quite suitable.

In summary, we have provided evidence that a low level of H$_2$O$_2$, in particular, a 20 μM level, can promote corneal epithelial cells migration and wound healing by enhancing Src activity on integrin engagement.

Acknowledgments

References

1. Cejkova J, Labsky J, Vacik J. Reactive oxygen species (ROS) generated by xanthine oxidase in the corneal epithelium and their potential participation in the damage of the corneal epithelium after prolonged use of contact lenses in rabbits. *Acta Histochem.* 1998;100:171–184.

2. Cejkova J, Stipek S, Crkovska J, Ardan T. Changes of superoxide dismutase, catalase and glutathione peroxidase in the corneal epithelium after UVB rays. Histochemical and biochemical study. *Histol Histopathol.* 2000;15:1043–1050.

3. Chiarugi P, Pani G, Giannoni E, et al. Reactive oxygen species as essential mediators of cell adhesion: the oxidative inhibition of a FAK tyrosine phosphatase is required for cell adhesion. *J Cell Biol.* 2003;161:933–944.

4. Kheradmand F, Werner E, Tremble P, Symons M, Werb Z. Role of Rac1 and oxygen radicals in collagenase-1 expression induced by cell shape change. *Science.* 1998;280:898–902.

5. Moldovan L, Moldovan NI, Sohn RH, Parikh SA, Goldschmidt-Clermont PJ. Redox changes of cultured endothelial cells and actin dynamics. *Circ Res.* 2000;86:549–557.

6. Sundaresan M, Yu ZX, Ferrans VJ, Irani K, Finkel T. Requirement for generation of H$_2$O$_2$ for platelet-derived growth factor signal transduction. *Science.* 1995;270:296–299.

7. Lo YY, Cruz TF. Involvement of reactive oxygen species in cytokine and growth factor induction of c-fos expression in chondrocytes. *J Biol Chem.* 1995;270:11727–11730.

8. Bae YS, Kang SW, Seo MS, et al. Epidermal growth factor (EGF)-induced generation of hydrogen peroxide. Role in EGF receptor-mediated tyrosine phosphorylation. *J Biol Chem.* 1997;272:217–221.

9. Chen KC, Zhou Y, Xing K, Krysan K, Lou MF. Platelet derived growth factor (PDGF)-induced reactive oxygen species in the lens epithelial cells: the redox signaling. *Exp Eye Res.* 2004;78:1057–1067.

10. Huo Y, Qiu WY, Pan Q, Yao YF, Xing K, Lou MF. Reactive oxygen species (ROS) are essential mediators in epidermal growth factor (EGF)-stimulated corneal epithelial cell proliferation, adhesion, migration, and wound healing. *Exp Eye Res.* 2009;89:876–886.

11. Thannickal VJ, Fanburg BL. Reactive oxygen species in cell signaling. *Am J Physiol Lung Cell Mol Physiol.* 2000;279:L1005–L1028.

12. Chiarugi P, Cirri P. Redox regulation of protein tyrosine phosphatases during receptor tyrosine kinase signal transduction. *Trends Biochem Sci.* 2003;28:509–514.

13. Giannoni E, Buricchi F, Raugei G, Ramponi G, Chiarugi P. Intracellular reactive oxygen species activate Src tyrosine kinase during cell adhesion and anchorage-dependent cell growth. *Mol Cell Biol.* 2005;25:6391–6403.

14. Giannoni E, Buricchi F, Grimaldi G, et al. Redox regulation of anoikis: reactive oxygen species as essential mediators of cell survival. *Cell Death Differ.* 2008;15:867–878.

15. Tatosyan AG, Mizenina OA. Kinases of the Src family: structure and functions. *Biochemistry (Mosc).* 2000;65:49–58.

16. Bertagnolli ME, Hudson LA, Stetsenko GY. Selective association of the tyrosine kinases Src, Fyn, and Lyn with integrin-rich actin cytoskeletons of activated, nonaggregated platelets. *Biochem Biophys Res Commun.* 1999;260:790–798.

17. Mazie AR, Spix JK, Block ER, Achebe HB, Klarlund JK. Epithelial cell motility is triggered by activation of the EGF receptor through phosphatidic acid signaling. *J Cell Sci.* 2006;119:1645–1654.

18. Block ER, Klarlund JK. Wounding sheets of epithelial cells activates the epidermal growth factor receptor through distinct short- and long-range mechanisms. *Mol Biol Cell.* 2008;19:4909–4917.

19. Xu KP, Yin J, Yu FS. SRC-family tyrosine kinases in wound- and ligand-induced epidermal growth factor receptor activation in human corneal epithelial cells. *Invest Ophthalmol Vis Sci.* 2006;47:2832–2839.

20. Peden DB, Dailey L, DeGraff W, et al. Hydrogen peroxide effects on rat mast cell function. *Am J Physiol.* 1994;267:L85–L93.

21. Dronadula N, Liu Z, Wang C, Cao H, Rao GN. STAT-3-dependent cytosolic phospholipase A2 expression is required for thrombin-induced vascular smooth muscle cell motility. *J Biol Chem.* 2005;280:3112–3120.

22. Henzler T, Steudle E. Transport and metabolic degradation of hydrogen peroxide in Chara corallina: model calculations and measurements with the pressure probe suggest transport of H(2)O(2) across water channels. *J Exp Bot.* 2000;51:2053–2066.

23. Murakami J, Nishida T, Otori T. Coordinated appearance of beta 1 integrins and fibronectin during corneal wound healing. *J Lab Clin Med.* 1992;120:86–93.

24. Fujikawa LS, Foster CS, Gipson IK, Colvin RB. Basement membrane components in healing rabbit corneal epithelial wounds: immunofluorescence and ultrastructural studies. *J Cell Biol.* 1984;98:128–138.

25. Suda T, Nishida T, Ohashi Y, Nakagawa S, Manabe R. Fibronectin appears at the site of corneal stromal wound in rabbits. *Curr Eye Res.* 1981;1:553–556.

26. Cai X, Foster CS, Liu JJ, et al. Alternatively spliced fibronectin molecules in the wounded cornea: analysis by PCR. *Invest Ophthalmol Vis Sci.* 1993;34:3585–3592.

27. Gao C, Negash S, Guo HT, Ledee D, Wang HS, Zelenka P. CDK5 regulates cell adhesion and migration in corneal epithelial cells. *Mol Cancer Res.* 2002;1:12–24.

28. Yin J, Lu J, Yu FS. Role of small GTPase Rho in regulating corneal epithelial wound healing. *Invest Ophthalmol Vis Sci.* 2008;49:900–909.

29. Westhoff MA, Serrels B, Fincham VJ, Frame MC, Carragher NO. SRC-mediated phosphorylation of focal adhesion kinase couples actin and adhesion dynamics to survival signaling. *Mol Cell Biol.* 2004;24:8113–8133.

30. Gao CY, Stepp MA, Fariss R, Zelenka P. Cdk5 regulates activation and localization of Src during corneal epithelial wound closure. *J Cell Sci.* 2004;117:4089–4098.

31. Xu KP, Ding Y, Ling J, Dong Z, Yu FS. Wound-induced HB-EGF ectodomain shedding and EGFR activation in corneal epithelial cells. *Invest Ophthalmol Vis Sci.* 2004;45:813–820.

32. Sundaresan M, Yu ZX, Ferrans VJ, et al. Regulation of reactive-oxygen-species generation in fibroblasts by Rac1. *Biochem J.* 1996;318(pt 2):379–382.

33. Archer H, Bar-Sagi D. Ras and Rac as activators of reactive oxygen species (ROS). *Methods Mol Biol.* 2002;189:67–73.

34. Moro L, Venturino M, Bozzo C, et al. Integrins induce activation of EGF receptor: role in MAP kinase induction and adhesion-dependent cell survival. *EMBO J.* 1998;17:6622–6632.

35. Biscardi JS, Ishizawar RC, Silva CM, Parsons SJ. Tyrosine kinase signalling in breast cancer: epidermal growth factor receptor and c-Src interactions in breast cancer. *Breast Cancer Res.* 2000;2:203–210.

36. Alexandrova AY, Kopnin PB, Vasiliev JM, Kopnin BP. ROS up-regulation mediates Ras-induced changes of cell morphology and motility. *Exp Cell Res.* 2006;312:2066–2073.

37. Haendeler J, Tischler V, Hoffmann J, Zeiher AM, Dimmeler S. Low doses of reactive oxygen species protect endothelial cells from apoptosis by increasing thioredoxin-1 expression. *FEBS Lett.* 2004;577:427–433.

38. Obenberger J, Cejkova J. Corneal damage following intracorneal injection of N-acetyl-L-cysteine. *Graefes Arch Klin Exp Ophthalmol.* 1972;185:171–176.

39. Thermes F, Molon-Noblot S, Grove J. Effects of acetylcysteine on rabbit conjunctival and corneal surfaces. A scanning electron microscopy study. *Invest Ophthalmol Vis Sci.* 1991;32:2958–2963.

40. Xu KP, Li Y, Ljubimov AV, Yu FS. High glucose suppresses epidermal growth factor receptor/phosphatidylinositol 3-kinase/Akt signaling pathway and attenuates corneal epithelial wound healing. *Diabetes.* 2009;58:1077–1085.

41. Brownlee M. The pathobiology of diabetic complications: a unifying mechanism. *Diabetes.* 2005;54:1615–1625.

42. Ohashi Y, Motokura M, Kinoshita Y, et al. Presence of epidermal growth factor in human tears. *Invest Ophthalmol Vis Sci.* 1989;30:1879–1882.