Hydrogen and *N*-Acetyl-L-Cysteine Rescue Oxidative Stress-Induced Angiogenesis in a Mouse Corneal Alkali-Burn Model

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Purpose. To investigate the role of reactive oxygen species (ROS) as the prime initiators of the angiogenic response after alkali injury of the cornea and observe the effects of antioxidants in preventing angiogenesis.

Methods. The corneal epithelia of SOD-1–deficient mice or wild-type (WT) mice were removed after application of 0.15 N NaOH to establish the animal model of alkali burn. ROS production was semiquantitatively measured by dihydroethidium (DHE) fluorescence. Angiogenesis was visualized by CD31 immunohistochemistry. The effects of the specific NF-κB inhibitor DHMEQ, the antioxidant N-acetyl-L-cysteine (NAC), and hydrogen (H_2) solution were observed.

Results. ROS production in the cornea was enhanced immediately after alkali injury, as shown by increased DHE fluorescence ($P < 0.01$). NF-κB activation and the upregulation of vascular endothelial growth factor (VEGF) and monocyte chemoattractant protein-1 (MCP-1) were significantly enhanced ($P < 0.01$), leading to a significantly larger area of angiogenesis. Angiogenesis in SOD-$1^{-/-}$ mice corneas were significantly higher in WT mice ($P < 0.01$), confirming the role of ROS. Pretreatment with the specific NF-κB inhibitor DHMEQ or the antioxidant NAC significantly reduced corneal angiogenesis by downregulating the NF-κB pathway ($P < 0.01$) in both WT and SOD-$1^{-/-}$ mice. Furthermore, we showed that irrigation of the cornea with hydrogen (H_2) solution significantly reduced angiogenesis after alkali-burn injury ($P < 0.01$).

Conclusions. Immediate antioxidant therapy with H_2-enriched irrigation solution is a new potent treatment of angiogenesis in cornea to prevent blindness caused by alkali burn. (*Invest Ophthalmol Vis Sci.* 2011;52:427–433) DOI:10.1167/iovs.10-6167

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Although the cornea is a physiologically transparent tissue, pathologic corneal neovascularization (CNV) is observed after inflammation caused by infection, aberrant immune responses, or chemical or thermal burns. The balance between angiogenic and antiangiogenic factors determines the fate of CNV during wound healing.[1–4] It is widely known that alkali burns cause acute inflammation and neovascularization in the cornea, and alkali burns in animals are often used as a neovascularization model.[5,6] However, the mechanisms involved in the induction of CNV are complex, with a long list of angiogenic growth factors such as fibroblast growth factor, vascular endothelial growth factor (VEGF), and transforming growth factor, in addition to chemokines that are mobilized after injury to the cornea.[1] Therefore, present therapeutic strategies for CNV have focused on inhibiting VEGF, the key trigger of pathologic CNV,[7] or blocking its receptors.[6] However, from a therapeutic standpoint, initial therapy to prevent the onset of CNV is far more effective and efficient. We therefore hypothesized that oxidative stress due to reactive oxygen species (ROS) at the onset of injury can be an upstream target for therapeutic intervention.

ROS include highly reactive molecules such as the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($\cdot$OH). ROS play an important role in cellular homeostasis, whereas pathologically high concentrations are involved with cell death, disease, and aging. ROS can activate the transcription factor NF-κB,[8] which then translocates to the nucleus to induce the expression of inflammatory cytokines such as VEGF, MCP-1, IL, and TNFα.[9] These cytokines not only induce CNV but can also recruit inflammatory cells that further exacerbate inflammation, causing further tissue damage. In this study, we demonstrate how ROS can directly trigger pathologic CNV in an animal model and, more importantly, show that the topical use of H_2 water has a prophylactic effect against pathologic blood vessels invading the clear cornea.

Materials and Methods

Alkali Burn Model

Male ICR mice at the age of 6 to 7 weeks were purchased from CLEA Japan, Inc. (Tokyo, Japan), and SOD-1–deficient mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the study was approved by the institutional board on the use of animals. CNV was induced by alkali injury. Briefly, after general anesthesia with pentobarbital (Nembutal, 50 mg/kg; Abbott Pharmaceutical, Abbott Park, IL), 2 μL of 0.15 M NaOH was applied to

the corneal surface. Subsequently, total corneal limbus and epithelium were scraped off with a surgical blade under a microscope. Ofloxacin ophthalmic ointment was instilled immediately after the operation.

Treatment with NAC or DHMEQ

From 3 days before the alkali burn, WT and SOD-1$^{-/-}$ mice were treated with an *N*-acetyl-L-cysteine (NAC; Nakarai tesque, Kyoto, Japan) or a specific NF-κB inhibitor (dehydroxymethylepoxyquinomicin [DHMEQ]) or vehicle (0.083% dimethyl sulfoxide [DMSO] in phosphate-buffered saline [PBS]) daily for 6 days. NAC and DHMEQ were injected into mice intraperitoneally. DHMEQ is a novel NF-κB inhibitor, based on the structure of epoxyquinomicin C, originally isolated from *Amycolatopsis*. DHMEQ has been shown to inhibit nuclear translocation of NF-κB without affecting the phosphorylation and degradation of I-κBα. Mice received NAC at a dose of 200 mg/kg or DHMEQ at 5 mg/kg body weight.

Treatment with H_2 Water

To observe the prophylactic effects of H_2 water, a potent antioxidant found effective in the prevention of ischemic brain injury,[10] eyes after alkali burns were irrigated with H_2 water (Blue Mercury Inc., Tokyo, Japan) for 30 minutes. H_2 water was rendered isotonic by diluting 20× concentrated PBS 1:20 before irrigation; the final H_2 concentration was in the range of 0.5 to 0.6 ppm. PBS alone was used as vehicle treatment, and untreated corneas were used as control.

Quantification of Corneal Angiogenesis

Six days after alkali burn, mice were killed. Enucleated eyes were further fixed with ice-cold 0.5% paraformaldehyde (PFA) for 10 minutes, followed by ice-cold methanol treatment at 70% for 20 minutes, 80% for 20 minutes, and 100% for 20 minutes. After washing steps in PBS and blocking with TNB buffer (1 N Tris-HCl [pH 7.5] 10 mL, DW 90 mL, NaCl 0.87 g, blocking reagent 0.5 g [FP1020; Perkin Elmer Life Sciences, Inc., Waltham, MA], 20% Triton 0.5 mL) in 0.1% Triton for 2 hours, whole corneas were stained overnight at 4°C with purified rat anti–mouse CD31 (PECAM-1) (1:300, Becton Dickinson, Franklin Lakes, NJ), washed, and further incubated with AlexaFluor 488 goat anti–rat IgG (1:300; Life Technologies, Carlsbad, CA) for 1 hour at room temperature. The flat mounts were imaged with a fluorescence microscope (Biorevo BZ-9000; Keyence, Osaka, Japan) and quantified by ImageJ software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov/ij/index.html). The area of CNV was calculated by the following equation in which the Total Area is the area within the limbal vessel arcade and the Avascular Area is the area of the remaining clear cornea: Area of Neovascularization (%) = (Total Area − Avascular Area)/Total Area × 100.

Measurement of ROS

Eyes were enucleated and immediately frozen in OCT compound (Sakura Finetek, Torrance, CA) as soon as 0.15 N NaOH dropped and washed out. Unfixed cryosections (10 μm) were incubated with 5 μM DHE (Molecular Probes, Eugene, OR) for 15 minutes at 37°C, as previously reported.[11,12] Sections were examined using a microscope equipped with a digital camera (Carl Zeiss, Oberkochen, Germany), and the intensity of the staining was measured using the ImageJ program. To facilitate the detection of ROS by image analysis, the epithelium was left intact for this assay.

Immunohistochemical Staining

Eyes were enucleated and fixed in 4% PFA overnight at 4°C. After fixation, tissues were processed and embedded in an OCT compound, frozen in liquid nitrogen, and stored at −80°C until sectioning. Frozen 6-μm to 8-μm–thick sections were cut with a cryostat and mounted on slides. We used the anti–mouse CD31 antibody (rat monoclonal, clone MEC 13.3, 550274; BD PharMingen, San Diego, CA) for detecting blood vessels in the cornea, or anti–mouse F4/80 antibody (rat monoclonal, clone CI: A3–1, MCA497R; AbD Serotec, Raleigh, NC) for macrophages. AlexaFluor 488- or 546-conjugated goat anti–rat antibodies were used as secondary antibody. Nuclei were counterstained with Hoechst 33342. The increase in the number of F4/80-positive cells per field was calculated at days 0, 3, and 6 after injury.

Enzyme-Linked Immunosorbent Assay

Three days after alkali burn, mice were killed with an overdose of anesthesia, and the eyes were immediately enucleated. The whole cornea, including the limbus, epithelium, stroma, and endothelium, was isolated and placed into 100 μL lysis buffer (0.02 M HEPES [4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid], 10% glycerol, 10 mM $Na_4P_2O_7$, 100 μM Na_3VO_4, 1% Triton, 100 mM NaF, 4 mM EDTA [EDTA], pH 8.0), supplemented with protease inhibitors (2 mg/L aprotinin, 100 μM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 2.5 μM pepstatin A), and sonicated. The lysate was centrifuged at 15,000 rpm for 15 minutes at 4°C. VEGF and MCP-1 levels in the supernatant were determined with the mouse VEGF and MCP-1 ELISA kits (R&D Systems, Minneapolis, MN), respectively, according to the manufacturer's protocols. Similarly, phosphorylated NF-κB p65 levels

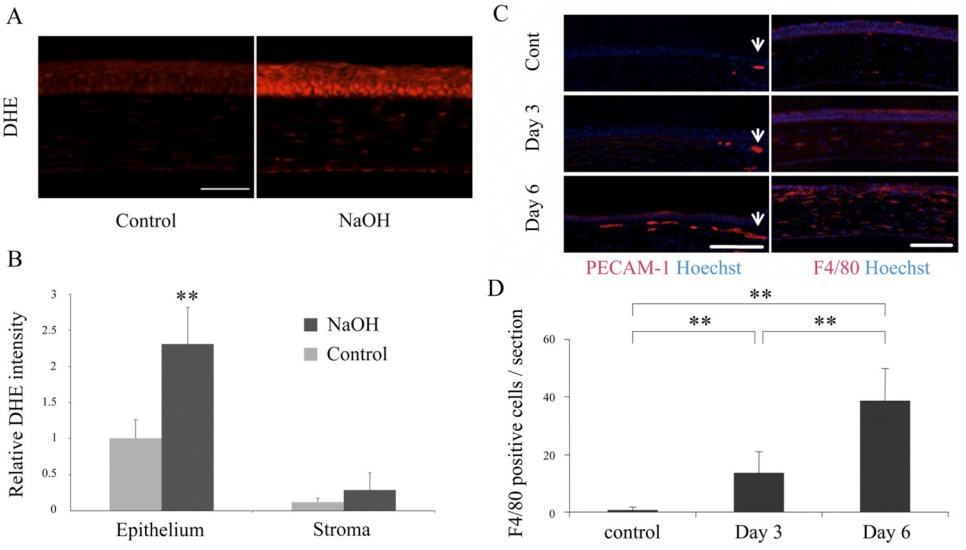

FIGURE 1. Alkali burns induce ROS formation. (**A**) Sodium hydroxide (0.15 N NaOH) induced oxidative stress in mice corneas, as shown by increased DHE fluorescence compared with controls. Scale bar, 100 μm. (**B**) DHE fluorescence levels in the cornea were significantly higher in alkali-burned mice (2.31 ± 0.51-fold of control; $^{**}P < 0.01$; $n = 4$). (**C**) Alkali burn induced immunoreactivity against PECAM-1 and F4/80, indicating angiogenesis and macrophage invasion. *Arrow*: normal vessels of the peripheral cornea. (**D**) F4/80-positive macrophages were significantly higher at day 3 (13.7 ± 7.4 cells/field; $P < 0.05$; $n = 6$) and day 6 (38.6 ± 11.4 cells/field; $P < 0.05$; $n = 5$) compared with day 0 (0.8 ± 0.8 cells/field). Error bars indicate SD. Scale bar, 100 μm.

were measured with the phosphorylated NF-κB p65 ELISA kit (Cell Signaling Technology, Danvers, MA) according to the manufacturer's instructions. The tissue sample concentration was calculated from a standard curve and corrected for protein concentration evaluated with a spectrophotometer (NanoDrop ND-1000; Thermo Fisher Scientific, Waltham, MA).

Quantitative RT-PCR

Animals were killed with an overdose of anesthesia. The eyes of each were immediately enucleated, and the cornea (including epithelium, stroma, endothelium, and limbus) was carefully isolated. Total RNA was extracted from the cornea using an extraction reagent (TRIzol; Life Technologies, Grand Island, NY), and cDNA was synthesized (First-Strand c-DNA Synthesis Kit; GE Health Care, Piscataway, NJ). For the RT-PCR reaction, mRNA transcripts were detected using the TaqMan real-time quantitative RT-PCR procedure (TaqMan Fast Universal PCR Master Mix Reagents Kit; Applied Biosystems, Foster City, CA). The RT-PCR assay was performed with a PCR system (7500 Fast Real-Time PCR; Applied Biosystems). The threshold cycle (Ct) was calculated by the instrument's software (7500 Fast; Applied Biosystems). A quantitative PCR assay for mRNA of f4/80 and mcp-1 was performed using gene expression master mix (TaqMan Gene Expression Assay Mix; Mm00802530_m1 and Mm00441242_m1, respectively; Applied Biosystems).

Statistical Analysis

All results were expressed as mean ± SD. Values were processed for statistical analyses (Mann-Whitney *U* test) or unpaired Student's *t*-test, and differences were considered statistically significant at $P < 0.05$.

Results

Alkali Burn Enhances Oxidative Stress in the Cornea

To elucidate the contribution of ROS in alkali-burned injury, we examined ROS induction by the administration of NaOH using the DHE assay, where DHE reacts with O_2^- to become fluorescent. Application of 0.15 N NaOH to the cornea induced a significant increase of ROS in the epithelium (2.31 ± 0.51-fold of untreated control; $P < 0.01$; Figs. 1A, 1B). Alkali burn induced angiogenesis (PECAM-1) and recruited macrophages (F4/80), which were not observed in untreated controls (Fig. 1C). F4/80-positive macrophages significantly increased by day 3 and day 6 ($P < 0.05$).

Alkali Burn-Induced CNV Was Inhibited by the Antioxidant NAC or the NF-κB Inhibitor DHMEQ

Increased oxidative stress in the alkali-burned cornea suggested that oxidative stress induced by alkali injury was the

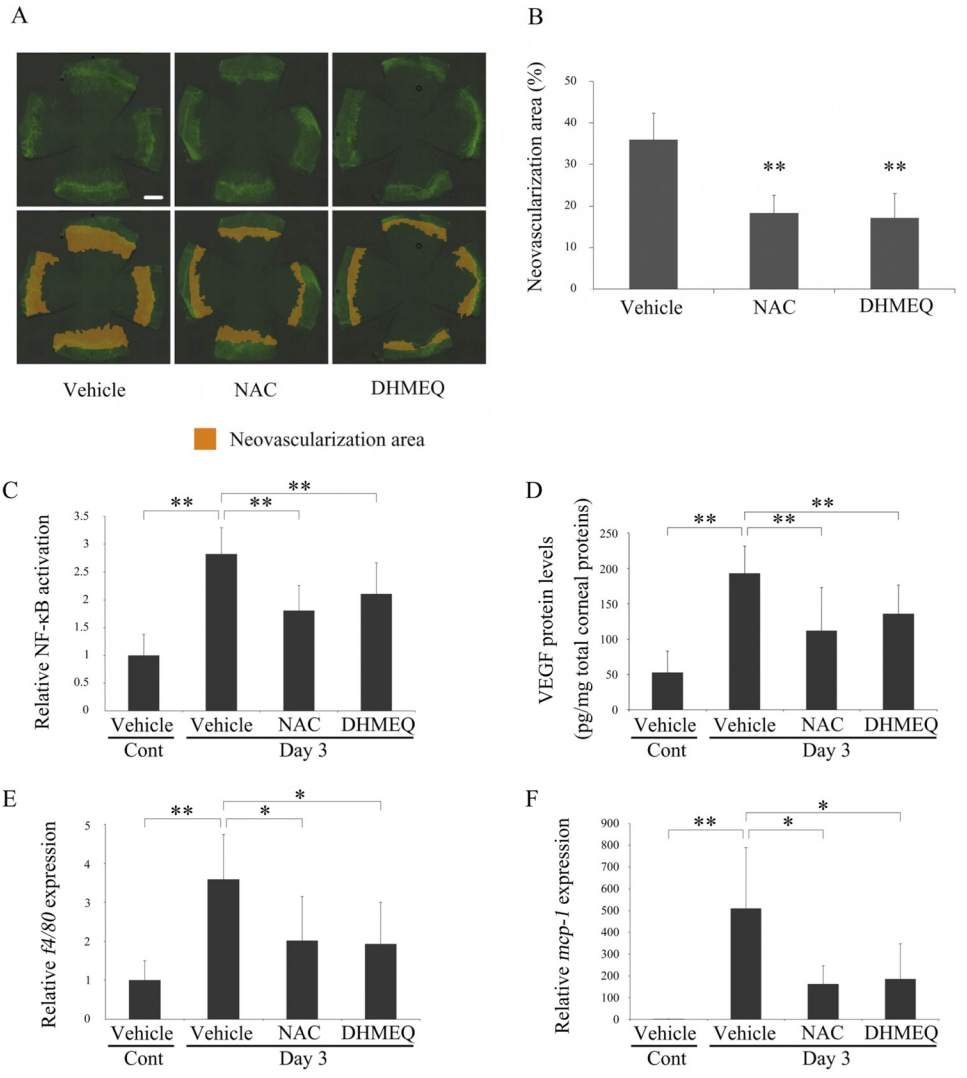

Figure 2. CNV caused by alkali burns is suppressed by NAC or DHMEQ. (**A**, **B**) Alkali burn–induced CNV in mice cornea. Neovascularization area was significantly (**$P < 0.01$ each) lower in NAC- or DHMEQ-treated mice (18.31 ± 4.25, 17.06 ± 5.99; $n = 8$) than in vehicle-treated controls (35.98 ± 6.35; $n = 6$; day 6). Scale bar, 500 μm. (**C**) NF-κB activation was significantly higher in alkali-burned mice, which was significantly suppressed by treatment with NAC or DHMEQ. $n = 8$–12 (day 3). **$P < 0.01$. (**D**) Similarly, VEGF protein levels were significantly elevated in alkali-burned mice, which were significantly suppressed by treatment with NAC or DHMEQ. $n = 8$–11 (**$P < 0.01$ each; day 3). *f4/80* (**E**) and *mcp-1* (**F**) mRNA in alkali-burned mice was significantly elevated compared with controls, which was significantly suppressed by treatment with NAC or DHMEQ. $n = 7$–8 and 8–9 for *f4/80* (*$P < 0.05$ each) and *mcp-1* (*$P < 0.05$ each), respectively. Error bars indicate SD.

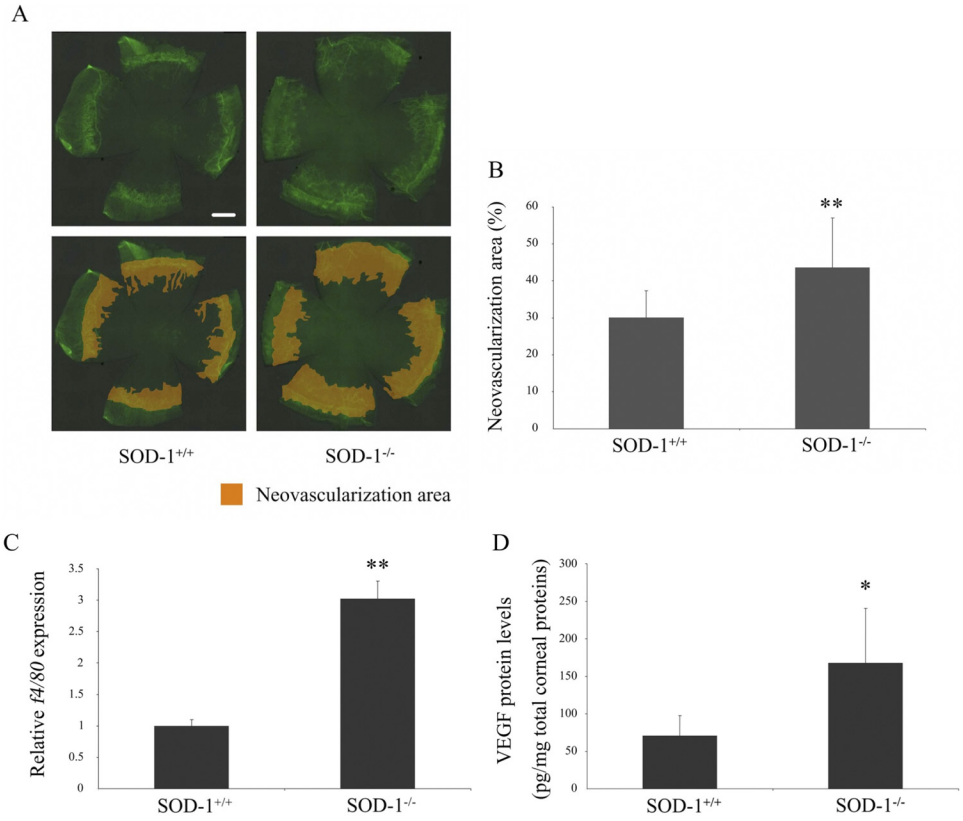

FIGURE 3. CNV is enhanced in SOD-$1^{-/-}$ mice. (**A**, **B**) CNV area was significantly (**$P < 0.01$) higher in SOD-$1^{-/-}$ mice (43.63 ± 13.42) than in SOD-$1^{+/+}$ mice (30.11 ± 7.16; n = 19 each; day 6). Scale bar, 500 μm. (**C**) *f4/80* mRNA levels in SOD-$1^{-/-}$ mice were significantly elevated compared with those in SOD-$1^{+/+}$ mice (n = 4 each; day 6) **$P <$ 0.01. (**D**) VEGF protein levels in SOD-$1^{-/-}$ mice were significantly higher than those in SOD-$1^{+/+}$ mice (n = 5–6; *$P < 0.05$; day 3). Error bars indicate SD.

main trigger for corneal angiogenesis. Therefore, we examined CNV in mice treated with the antioxidant NAC. As expected, antioxidant treatment significantly suppressed alkali-burn induced CNV ($P < 0.01$). Furthermore, the inhibition of NF-κB, a transcription factor downstream of oxidative stress, suppressed CNV as well ($P < 0.01$; Figs. 2A, 2B).

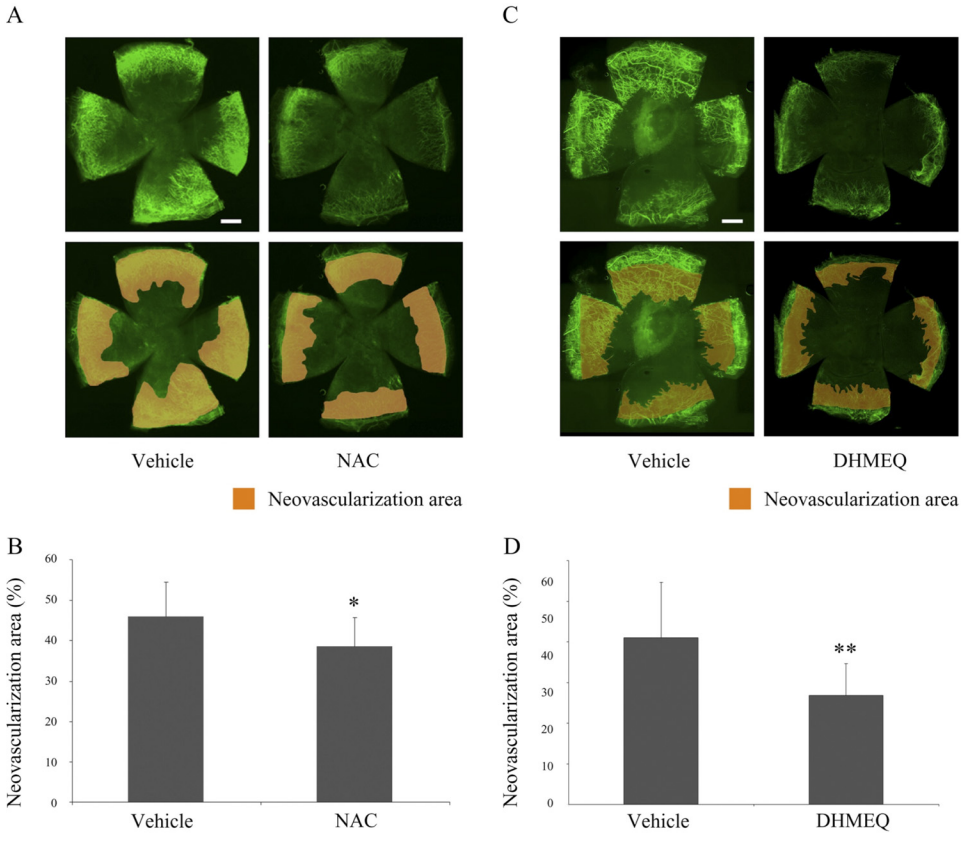

FIGURE 4. CNV in SOD-$1^{-/-}$ mice is suppressed by NAC and DHMEQ. (**A**, **B**) CNV area in SOD-$1^{-/-}$ mice was significantly lower than in control (45.85 ± 8.67; n = 11) when pretreated with NAC (38.53 ± 7.19; n = 13) starting 3 days before injury (*$P <$ 0.05). (**C**, **D**) A similar reduction in neovascularization area was observed in DHMEQ-treated mice (26.78 ± 7.81; n = 13) compared with vehicle-treated controls (43.626 ± 13.427; n = 19; **$P < 0.01$; day 6). Error bars indicate SD. Scale bar, 500 μm.

To investigate the molecular mechanisms involved, we examined NF-κB phosphorylation and VEGF expression in alkali-burned corneas. A statistically significant increase in NF-κB phosphorylation ($P < 0.01$; Fig. 2C) and VEGF expression ($P < 0.01$) was induced by alkali injury, which was inhibited by the administration of NAC or DHMEQ (Fig. 2D). CNV induced by various kinds of stimuli, including alkali burn, is accompanied with and accelerated by infiltrating macrophages.[13] To quantify macrophage infiltration to the cornea, we measured mRNA levels of the macrophage marker f4/80. We found that f4/80 mRNA in alkali-burned corneas 6 days after injury decreased with NAC ($P < 0.05$) or DHMEQ ($P < 0.05$) treatment (Fig. 2E). Similarly, alkali burn-induced increase of mcp-1, a chemotactant for macrophage infiltration,[14] was also suppressed by NAC ($P < 0.05$) or DHMEQ ($P < 0.05$; Fig. 2F). These results indicated antioxidant treatment or inhibition of NF-κB suppressed alkali-burned CNV by the downregulation of VEGF and the suppression of macrophage infiltration.

SOD-1$^{-/-}$ Mice Show Enhanced CNV

To confirm the role of ROS in corneal angiogenesis induced by alkali burn, we compared neovascularization areas in SOD-1$^{-/-}$ mice with littermate WT controls. The neovascularization area in SOD-1$^{-/-}$ mice (43.63% ± 13.42%, $n = 6$) was significantly larger than in SOD-1$^{+/+}$ control (30.11% ± 7.16%; $n = 6$; $P < 0.01$; Figs. 3A, 3B). This shows that the loss of SOD-1 function enhanced alkali burn-induced CNV. The expression of *f4/80* was significantly increased in SOD-1$^{-/-}$ compared with SOD-1$^{+/+}$ ($P < 0.01$; Fig. 3C). Furthermore, VEGF protein was significantly increased in SOD-1$^{-/-}$ compared with SOD-1$^{+/+}$ ($P < 0.05$; Fig. 3D). These results suggest that the antioxidant effect of SOD-1 suppresses corneal angiogenesis by inhibiting macrophage infiltration and VEGF upregulation after alkali burns.

NAC and DHMEQ Rescues Increased CNV in SOD-1$^{-/-}$ Mice

Because the loss of SOD-1 expression caused an increase in macrophage infiltration, VEGF expression, and neovascularization, we examined whether NAC can rescue the phenotype and whether NF-κB was involved. As expected, we found that both NAC (Figs. 4A, 4B; $P < 0.05$) and DHMEQ (Figs. 4C, 4D) significantly decreased the neovascularization area ($P < 0.01$), indicating that the NF-κB pathway was activated by the increase in ROS associated with the loss of SOD-1 protein.

Post-Trauma Irrigation with H_2 Water Suppresses CNV

We found that pretreatment with NAC can prevent neovascularization formation in the cornea. Given that pretreatment is not possible in the clinical setting, we further sought to find a rescue protocol that is effective after alkali injury. The most important emergency procedure after corneal chemical burns is meticulous irrigation of the ocular surface using saline solutions to dilute residual chemicals in the tissue. We therefore hypothesized that irrigating with H_2 water, a recently recognized antioxidant, instead of saline will enhance the prophylactic effects against ensuing inflammation and CNV. NAC was not used in the irrigation experiment because of its acidic pH. As shown in Figure 5, irrigating with isotonic H_2 water significantly reduced ROS-associated DHE fluorescence ($P < 0.05$; Figs. 5A, 5B) and NF-κB phosphorylation ($P < 0.05$; Fig. 5C). More important, eyes irrigated with H_2 water also had significantly smaller neovascularization areas ($P < 0.01$; Figs. 6A, 6B) that were associated with lower VEGF ($P < 0.01$; Fig. 6C) and MCP-1 ($P < 0.01$; Fig. 6D) protein levels compared with PBS alone.

Discussion

In the present study, we showed that oxidative stress triggered angiogenesis through activation of the NF-κB pathway using a corneal alkali burn model. The role of ROS was confirmed using SOD-1$^{-/-}$ mice, which showed increased VEGF protein levels and accumulation of macrophages that further accelerated CNV (Fig. 3). Although angiogenesis induced by alkali burn is not recognized as an oxidative stress model, we also examined CNV by ultraviolet B radiation (UVB), a more common source of oxidative stress. SOD-1$^{-/-}$ mice had signifi-

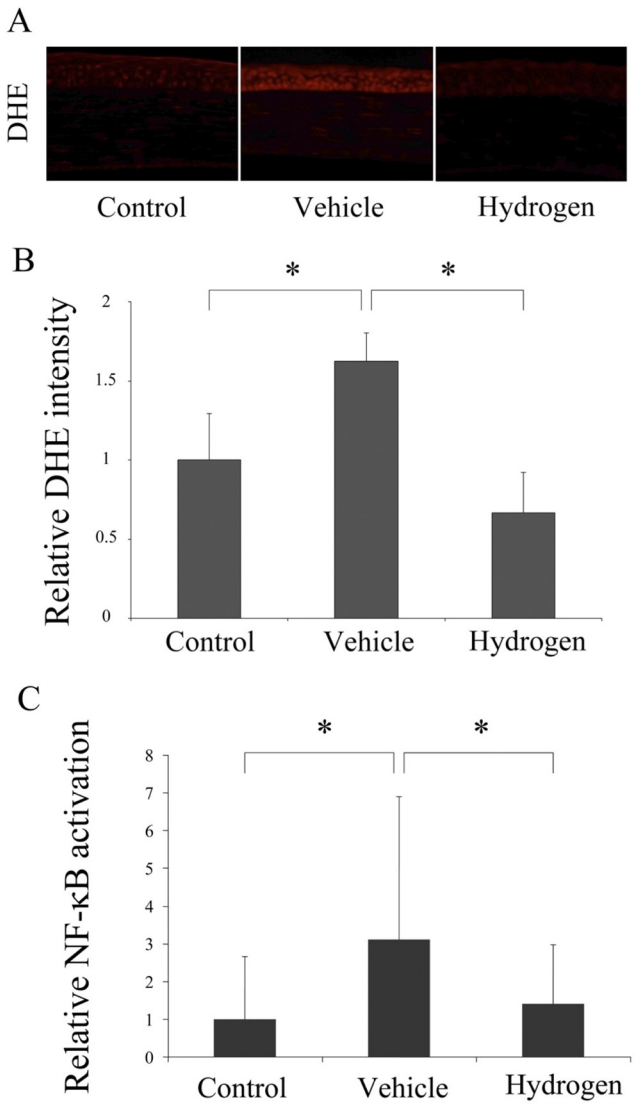

Figure 5. Corneal ROS formation is suppressed with H_2. To examine oxidative stress as a potential stimulus for angiogenic and molecular inflammatory events, corneal ROS expression was analyzed by DHE fluorescence. (**A**, **B**) ROS-induced DHE fluorescence in the corneal epithelium was significantly higher in vehicle-treated alkali-burned mice (1.6 ± 0.2 ratio of control; $^*P < 0.05$) than in untreated controls. DHE intensity was significantly suppressed in H_2-treated mice (0.6 ± 0.3 ratio of control; $^*P < 0.05$) than in vehicle-treated alkali-burned mice (1.6 ± 0.2 ratio of control; $n = 4$–5). Scale bar, 100 μm. (**C**) Phosphorylated NF-κB p65 levels were significantly ($^*P < 0.05$) higher in vehicle-treated alkali-burned mice (311% ± 381% of control) than in untreated controls (100% ± 176% of control). Administration of H_2 to alkali-burned animals significantly ($^*P < 0.05$) reduced phosphorylated NF-κB p65 levels (140% ± 158% of control; $n = 28$–29). Error bars indicate SD.

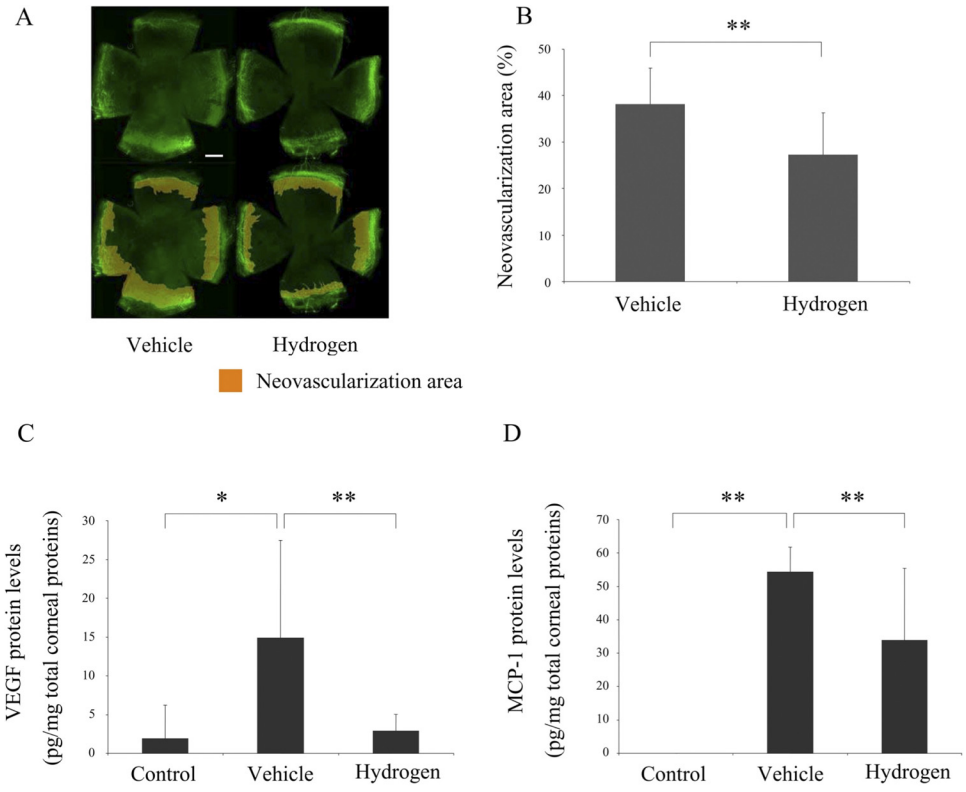

FIGURE 6. (**A**) Alkali burn–induced CNV is inhibited with H_2. Neovascularization area in the whole cornea was evaluated semiquantitatively. Scale bar, 500 μm. Neovascularization area was significantly lower ($^{**}P < 0.01$, **B**) in H_2-treated mice (27.3% ± 9.0%) than in vehicle-treated controls (38.1% ± 7.8%; n =13–15). Corneal VEGF (**C**) and MCP-1 (**D**) protein levels were significantly ($^{*}P < 0.05$ and $^{**}P < 0.01$, respectively) higher in vehicle-treated alkali-burned mice (14.9 ± 12.5 pg/mg and 54.4 ± 7.3 pg/mg, respectively) than in vehicle-treated controls (1.9 ± 4.3 pg/mg and nondetectable, respectively). Administration of H_2 to alkali-burned animals significantly ($^{**}P < 0.01$ for both) reduced VEGF and MCP-1 protein levels (2.9 ± 2.1 pg/mg and 34.0 ± 21.5 pg/mg, respectively; n =13–15). Error bars indicate SD.

cantly more CNV than WT control following exposure to UVB (Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-6167/-/DCSupplemental). The current understanding of the alkali burn model of angiogenesis is that inflammation is the trigger of angiogenesis after injury. Therefore, most studies have focused on anti-inflammatory agents such as NF-κB inhibitors,[5,13] anti–VEGF agents,[15,16] and inhibition of the VEGF receptor.[6] Corticosteroids and nonsteroidal anti-inflammatory drugs are also used clinically to suppress inflammation; however, these agents are nonspecific with side effects such as increased ocular pressure. We also examined the effects of the specific NF-κB inhibitor DHMEQ and confirmed that inhibiting NF-κB reduces VEGF protein levels, *mcp-1* expression, and neovascularization area after alkali burns (Fig. 2). Nuclear translocation of NF-κB upregulates *mcp-1* and *vegf*, leading to increased macrophage infiltration and angiogenesis, respectively.[9] Compared with other NF-κB inhibitors reported in the literature, DHMEQ is unique in that it blocks the translocation of NF-κB p65 into the nucleus[17] and is therefore highly specific to this pathway.

However, the most important finding of our study is the fact that direct ROS formation by alkali injury precedes the inflammatory response. ROS-related DHE fluorescence was enhanced immediately after alkali burn (Fig. 1A). Given that oxidative stress was upstream of the cascade leading to CNV, we hypothesized that supplementing exogenous antioxidants such as NAC may protect the cornea from alkali burn-induced angiogenesis. As expected, pretreatment with NAC significantly reduced the angiogenic response after alkali burns, as shown in Figure 2. NAC inhibited VEGF production, *f4/80* and *mcp-1* expression, and neovascularization comparable to the effects by DHMEQ, demonstrating the potency of ROS in the activation of NF-κB. Of particular note is the fact that NAC treatment was administered only up to 2 days after injury, indicating that early intervention with antioxidants was particularly important. Recent studies have also shown that oxidative stress promotes pathologic events by NF-κB activation[18,19] and that NF-κB is indeed a redox-sensitive transcriptional factor.[8,9]

Because pretreatment with antioxidants in not clinically feasible, we further investigated the possibility of using H_2 water as an antioxidant irrigation solution after injury. H_2 water irrigation significantly quenched ROS-related DHE fluorescence and NF-κB phosphorylation (Fig. 5). Furthermore, the 30-minute irrigation period was sufficient to significantly reduce VEGF and MCP-1 protein levels and the final area of CNV. H_2 gas is a harmless gas by itself and is used as a diluent gas for deep sea divers to improve ventilatory mechanics and to attenuate the excitatory effects of high pressure on the central nervous system.[20] Recently, Ohsawa et al.[10] introduced H_2 in the field of medicine as a specific inhibitor of the hydroxyl radical, a particularly reactive ROS. Hydrogen reduces the hydroxyl radical to H_2O_2, thereby preventing subsequent lipid peroxidation, DNA oxidation, and mitochondrial dysfunction. They reported that inhalation of H_2 in a rat model of cerebral ischemia and reperfusion model significantly reduced ROS-induced brain injury. Similar reports since then showed similar protective effects of H_2 against ROS damage in a neonatal rat model,[21] an intestinal graft injury model,[22] and a drug-induced nephrotoxicity model.[23] Unlike other antioxidants, H_2 can diffuse directly through tissue because of its small molecular size, and it does not require specific carriers or membrane channels.[10]

Chemical burn injuries of the cornea are caused by various toxic chemicals used primarily in the industry. Alkali injuries are the most severe, causing blindness in the working age population, with an incidence ranging from 7.0% to 9.9% of all ocular traumas.[24–26] Alkaline was the most common causative agent (66.7%) among chemical injuries in a series from the United Kingdom. [27] The incidence is probably higher in developing countries based on the number of surgical procedures performed.[28] Prompt irrigation of the injured eye at the site of injury and in the emergency clinic is vital to prevent blindness; advanced stages are more difficult to treat because of stem cell

deficiency and corneal angiogenesis. In conclusion, our study showed that oxidative stress is a direct result of alkali injury and that immediate action to quench ROS can inhibit pathologic angiogenesis. Furthermore, compared to using expensive reagents such as antibodies or antimetabolites, H_2 water is inexpensive and safe to use in any clinical setting. We hope our results will shed light on the importance of antioxidant therapy in alkali burns.

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