

Involvement of NADPH oxidases in alkali burn-induced corneal injury

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Received June 7, 2015; Accepted April 22, 2016

DOI: 10.3892/ijmm.2016.2594

Abstract. Chemical burns are a major cause of corneal injury. Oxidative stress, inflammatory responses and neovascularization after the chemical burn aggravate corneal damage, and lead to loss of vision. Although NADPH oxidases (Noxs) play a crucial role in the production of reactive oxygen species (ROS), the role of Noxs in chemical burn-induced corneal injury remains to be elucidated. In the present study, the transcription and expression of Noxs in corneas were examined by RT-qPCR, western blot analysis and immunofluorescence staining. It was found that alkali burns markedly upregulated the transcription and expression of Nox2 and Nox4 in human or mouse corneas. The inhibition of Noxs by diphenyleneiodonium (DPI) or apocynin (Apo) effectively attenuated alkali burn-induced ROS production and decreased 3-nitrotyrosine (3-NT) protein levels in the corneas. In addition, Noxs/CD11b double-immunofluorescence staining indicated that Nox2 and Nox4 were partially co-localized with CD11b. DPI or Apo prevented the infiltration of CD11b-positive inflammatory cells, and inhibited the transcription of inflammatory cytokines following alkali burn-induced corneal injury. In our mouse model of alkali burn-induced corneal injury, corneal neovascularization (CNV) occurred on day 3, and it affected 50% of the whole area of the cornea on day 7, and on day 14, CNV coverage of the cornea reached maximum levels. DPI or Apo effectively attenuated alkali burn-induced CNV and decreased the mRNA levels of angiogenic factors, including vascular endothelial growth factor (VEGF), VEGF receptors and matrix metalloproteinases (MMPs). Taken together, our data indicate that Noxs play a role in alkali burn-induced corneal injury by

regulating oxidative stress, inflammatory responses and CNV, and we thus suggest that Noxs are a potential therapeutic target in the future treatment of chemical-induced corneal injury.

Introduction

Chemical-induced corneal injury usually leads to extensive damage to the entire anterior segment of the eye, and remains a major cause of corneal diseases, which severely affects visual function and is not easily cured by conservative treatment (1). It has been well documented that acute oxidative stress, inflammation and corneal neovascularization play a crucial role in chemical burn-induced corneal damage and even loss of vision (2). Chemical burns stimulate the production of reactive oxygen species (ROS) in the corneas (3). When ROS production is low or intermediate, oxidative stress is prevented by intracellular antioxidant systems, such as superoxide dismutase (SOD), catalase, glutathione peroxidases and peroxiredoxins. ROS actively participate in various cellular processes, such as cell proliferation, differentiation and inflammation (4). However, if ROS production overwhelms the cellular antioxidant capacity, the high level of oxidative stress induces the perturbation of the mitochondrial permeability transition pore and disrupts electron transfer, eventually leading to apoptosis or necrosis. It has been demonstrated that high-level oxidative stress contributes to the pathogenesis of several diseases, such as diabetes (5,6), age-related macular degeneration (7) and chemical injury of the cornea (8). On the other hand, ROS act as second messengers in triggering inflammation through the activation of nuclear factor- κ B (NF- κ B), and subsequently stimulate the release of inflammatory cytokines (9), the expression of vascular endothelial growth factor (VEGF) (10) and matrix metalloproteinases (MMPs) (11). The inflammatory responses, and VEGF and MMPs, play an important role in pathologic corneal neovascularization (CNV) after chemical burns. In general, ROS are mainly generated through the mitochondrial electron transport chain and enzymes, such as NADPH oxidases (Noxs). Although the contribution of ROS to chemical burn-induced inflammation and pathological CNV has been previously recognized, the role of Noxs in chemical burn-induced corneal damage and CNV remains to be elucidated.

There are 7 identified isoforms in the Nox family, including Nox1, Nox2, Nox3, Nox4, Nox5, and dual oxidase 1 and 2

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Key words: NADPH oxidase, corneal injury, alkali burn, oxidative stress, inflammation, neovascularization

(DUOX1 and DUOX2) (12). According to a model of activation, the 7 members of the Nox family have been classified as constitutively active and acutely activated enzymes. Nox2 was first described in neutrophils and macrophages (13), and is also the member which has received the most scholarly attention (14). Nox2 is located on the plasma membrane, and forms a complex with the p22phox subunit. The activation of Nox2 requires combination with p47phox, p67phox, p40phox and Ras-related C3 botulinum toxin substrate 1 (Rac1) (15). By contrast, Nox4 is the single constitutively active member of the Nox family. Nox4 was originally identified in the kidneys (16), and is mainly expressed in vascular endothelial cells and smooth muscle cells (17,18). ROS are generated by Noxs through the transfer of electrons across biological membranes (13). It has previously been demonstrated that Noxs contribute to oxidative injury in endothelial cells in diabetic rats *in vivo* (19), as well as in the migration and proliferation of endothelial cells (20). Nox-mediated oxidative stress also plays a crucial role in the activation of the NF- κ B signaling pathway (21) and MMPs (22), which contributes to inflammatory responses and cell migration. In a previous study, it was found that the inhibition of Nox4 activity effectively decreased VEGF expression and retinal vascular permeability in *db/db* mice (23), suggesting that Nox4 is involved in retinal neovascularization. In addition, the expression of Noxs has been detected in corneal epithelial and stromal cells (24,25). However, the expression of Noxs in corneas after chemical burns and its role in corneal damage, inflammation and pathologic CNV remain unclear.

In the present study, we established a mouse model of alkali burn-induced corneal injury, and examined the expression of Noxs in human and mouse corneas after alkali burns. It was found that the expression of Nox2 and Nox4 was significantly increased in human and mouse corneas after alkali burns. Furthermore, ROS production, oxidative stress, inflammatory cytokine release and CNV were increased after alkali burns. The Nox inhibitors, diphenyleiiodonium (DPI), or apocynin (Apo) effectively attenuated alkali burn-induced oxidative stress, the activation of the inflammatory response and pathological CNV in the corneas. Therefore, our data indicate the vital role which Noxs play in alkali burn-induced injury to the corneas.

Materials and methods

Human corneal tissues. Normal human corneas and corneas which had been affected by alkali burns were obtained from the Affiliated Eye Hospital of Nanchang University, Nanchang, China. Normal corneas were obtained from the archives, and the corneas affected by alkali burns were obtained after penetrating keratoplasty. All experiments involving patients were approved by the Ethics Committee of the Affiliated Eye Hospital of Nanchang University and were performed in accordance with the principles of the Declaration of Helsinki. The corneal tissues were conventionally fixed with 4% paraformaldehyde. The slices of the cornea which were embedded in optimal cutting temperature (OCT) compound (Sakura Finetek, Inc., Torrance, CA, USA) were subsequently prepared at 7 μ m thickness.

Animals. C57BL/6 mice (n=68, 6-8 weeks of age) were purchased from Hunan SJA Laboratory Animal Co., Ltd., (Hunan, China) and all experiments involving animals were approved

by the Shanghai Animal Institution, Chinese Academy of Sciences (Shanghai, China). The animal experiments were conducted in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research.

Establishment of the mouse model of alkali burn-induced corneal injury and treatment with Nox inhibitors. The animal model of alkali burn-induced corneal injury was established as previously described (26). Briefly, the mice were anesthetized by an intraperitoneal injection of 10% chloral hydrate (0.2 ml/100 g). Subsequently, 0.4% oxybuprocaine hydrochloride (Santen, Tokyo, Japan) was applied topically to the corneal surface. Filter paper (2.0 mm in diameter) soaked in 2 μ l of 1 N NaOH was placed on the central cornea of the right eye for 40 sec under a surgical microscope, and the eyes were then rinsed with 10 ml saline buffer. To examine the role of Noxs in alkali burn-induced corneal injury, two commonly used Nox inhibitors, DPI and Apo (Sigma-Aldrich, St. Louis, MO, USA), were administered by eye drops at concentrations previously described (23,27). The stock solution of DPI was prepared at 3 mM in DMSO, and diluted to 0.1 μ M in PBS for use. Apo was prepared at 500 mM and diluted to 500 μ M for use. The final DMSO concentration in each eye drop was <0.1%. Immediately after corneal injury, DPI or Apo were administered by eye drops and applied to the mouse corneas 4 times a day for 7 or 14 days. Saline with an equivalent concentration of DMSO was used as the control for treatment.

Immunofluorescence staining. The mice were sacrificed by cardiac injection of PBS before the eyes were removed for immunofluorescence staining. A total of 9 mice was used for this experiment. The eyes were removed 7 days after treatment, and were immediately frozen in OCT compound. The OCT-embedded slices of the cornea were prepared at 7 μ m thickness for immunofluorescence staining. The corneal sections were fixed in ice-cold acetone for 20 min and washed in PBS. After blocking in 1% BSA, immunofluorescence staining was performed with the following primary antibodies: rabbit anti-Nox4 (1:100; sc-30141; Santa Cruz Biotechnology, Santa Cruz CA, USA), mouse anti-gp91-phox (Nox2) (1:100; sc-130543; Santa Cruz Biotechnology), mouse anti-3-nitrotyrosine (3-NT) (1:200; ab1392; Abcam, Cambridge, MA, USA), rat anti-CD11b (1:10; M1/70.15.11.5.2; Hybridoma Bank, Iowa City, IA, USA). The secondary antibodies included the following: Alexa Fluor[®] 488 donkey anti-rabbit IgG (H+L) (1:200; A-21206; Invitrogen, Carlsbad, CA, USA), Alexa Fluor[®] 488 donkey anti-mouse IgG (H+L) (1:200; A-21202; Invitrogen), Alexa Fluor[®] 594 rabbit anti-mouse IgG (H+L) (1:200; A27027; Invitrogen), Alexa Fluor[®] 594 donkey anti-rat IgG (H+L) (1:200; A-21202; Invitrogen). After 3 washes with TBST, the sections were further incubated with mounting medium with DAPI (H-1200; Vector Laboratories, Burlingame, CA, USA). Fluorescence signals were detected under a fluorescence microscope (Olympus, Tokyo, Japan).

Reverse transcription quantitative PCR (RT-qPCR). Total RNA was extracted from the corneas of 24 mice using ice-cold TRIzol reagent (Invitrogen) and 500 ng total RNA was reverse transcribed for the synthesis of double-stranded cDNA using TransScript[®] (AE301; Beijing Transgen Biotech Co., Ltd., Beijing, China). Real-time (quantitative) amplification was

performed using SsoFast™ EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA, USA) in a CFX connect real-time PCR system (Bio-Rad Laboratories). PCR reactions were carried out at 25°C for 10 min and 42°C for 30 min, 95°C for 30 sec, and then 40 cycles at 95°C for 5 sec, 60°C for 5 sec. The primers used for qPCR were as follows: Nox1, 5'-TGGCTAAATCCCATCCAGTC-3' (forward) and 5'-CCCAAGCTCTCCTCTGTTT-3' (reverse); Nox2, 5'-TCGCTGGAACCCCTCCTATG-3' (forward) and 5'-GGATACCTTGGGCACTTGA-3' (reverse); Nox4, 5'-ACTTTTCATTGGGCGCTC-3' (forward) and 5'-AGAAGTGGGTCACAGCAGA-3' (reverse); peptidylprolyl isomerase A (PPIA), 5'-AATGCTGGACCAACACAAA-3' (forward) and 5'-TTCACAATGTTCATGCCTT-3' (reverse); tumor necrosis factor- α (TNF- α), 5'-CAGCCTCTTCTCATTCTGCTTG-3' (forward) and 5'-GGGTCTGGGCCATAGAAGTGA-3' (reverse); interleukin (IL)-1 β , 5'-CTCCATGAGCTTTGTA CAAGG-3' (forward) and 5'-TGCTGATGTACCAGTTGGGG-3' (reverse); IL-6, 5'-CAAAGCCAGAGTCCTTCAGA-3' (forward) and 5'-GATGGTCTTGGTCCTTAGCC-3' (reverse); VEGF, 5'-TTACTGCTGTACCTCCACC-3' (forward) and 5'-ACAGGACGGCTTGAAGATG-3' (reverse); VEGF receptor (VEGFR)1, 5'-GTGATCAGCTCCAGGTTTGA CTT-3' (forward) and 5'-GAGGAGGATGAGGGTGTCTA TAGGT-3' (reverse); VEGFR2, 5'-CTGTGAACGCTTGCC TTAT-3' (forward) and 5'-CAACATCTTGACGGCTACTG-3' (reverse); MMP2, 5'-CCCCGATGCTGATACTGA-3' (forward) and 5'-CTGTCCGCCAAATAAACC-3' (reverse); MMP9, 5'-CAGCCAACTATGACCAGGAT-3' (forward) and 5'-CTGCCACCAGGAACAGG-3' (reverse); MMP13, 5'-GTGTGGAGTTATGATGATGT-3' (forward) and 5'-TGCGATTAC TCCAGATACTG-3' (reverse).

Measurement of corneal ROS production. Corneal ROS production was measured using CellROX Green reagent (Invitrogen) according to the manufacturer's instructions. In brief, the fresh corneal sections were washed with PBS and permeabilized in 0.5% Triton-X for 10 min. To detect ROS levels in the corneas, 5 μ M CellROX reagent were added to the sections followed by incubation for 30 min at 37°C. Following incubation, the sections were washed 3 times with PBS. Fluorescence signals of ROS were detected with excitation and emission wavelengths at 485/530 nm under a fluorescence microscope (Olympus), and fluorescence intensities were analyzed using ImageJ software (Broken Symmetry Software).

Assessment of CNV. To observe CNV following alkali burn-induced injury in our time course experiments, the mouse corneas from 15 mice were examined under a dissecting microscope (SM200L; Olympus) and photographed. In addition, FITC-dextran corneal angiography was used to quantify the area of CNV. Briefly, the mice were sacrificed 14 days after being subjected to alkali burns by a cardiac injection of FITC-dextran (Sigma-Aldrich). The eyes were removed and fixed in 4% paraformaldehyde at 4°C for 2 h. The corneas were excised and processed for whole-mount preparation. The CNV signal was detected under a fluorescence microscope (Olympus), and the positive CNV area was quantified using cellSens software (Olympus).

Western blot analysis. The whole lysate of the dissected corneal tissues from 20 mice was prepared using radio immunoprecipitation assay (RIPA) lysis buffer. Proteins in whole lysate were separated by 7.5% SDS-PAGE and transferred onto nitrocellulose membrane (HATF00010; Millipore, Bellerica, MA, USA). The membranes were blocked in 5% skim milk in TBST buffer for 1 h at room temperature, and then immunoblotted for 2 h at room temperature with the following primary antibodies: rabbit anti-Nox4 (sc-30141; Santa Cruz Biotechnology), mouse anti-gp91-phox (sc-130543; Santa Cruz Biotechnology), mouse anti-3-NT (ab1392; Abcam) and mouse anti β -actin (sc-47778; Santa Cruz Biotechnology). Following 3 washes with TBST, the membranes were further incubated with a horseradish peroxidase-conjugated secondary antibody (ZB-2310; Zhongshan Jinqiao, Beijing, China). Chemiluminescence assays were carried out with enhanced chemiluminescence reagents (SuperSignal® West Dura Extended Duration Substrate; Thermo Fisher Scientific, Waltham, MA, USA). The immunoblot signal was detected using ChemiFast (G:BOX Chemi XT4; Syngene International Ltd., San Jose, CA, USA), and the signal density of each band was analyzed using ImageJ software (Broken Symmetry Software).

Statistical analysis. The quantitative data are presented as the means \pm SD. Statistical analysis was performed using SPSS 17.0 software. Data were analyzed using one-way ANOVA or a Student's t-test to perform comparisons between 2 groups, and a P-value <0.05 was considered to indicate a statistically significant difference.

Results

Expression of Noxs in human and mouse corneas following alkali burn-induced corneal injury. It has been reported that alkali burns increase ROS generation, and trigger inflammatory responses and neovascularization in corneas (3). Noxs are an important source of ROS. Thus, to investigate the possible role of Noxs in alkali burn-induced oxidative stress and injury to corneas, the expression of Nox isoforms was examined. Immunofluorescence staining demonstrated that the expression of Nox2 and Nox4 was upregulated in both the human and mouse corneas affected by alkali burns (Fig. 1A-D). The results of western blot analysis also revealed the upregulation of Nox2 and Nox4 in the mouse corneas following alkali burn-induced injury (Fig. 1E and F). In addition, the mRNA level of Nox isoforms was measured by RT-qPCR. As shown in Fig. 1G, alkali burns induced the transcription of Nox2 and Nox4, but decreased the mRNA levels of NNox1 in mouse corneas. These results indicate that alkali burns selectively stimulate the transcription and expression of Nox2 and Nox4 in both human and mouse corneas, and we thus suggest that Nox2 and Nox4 are involved in alkali burn-induced oxidative stress and corneal damage.

Alkali burn-induced oxidative stress is attenuated by Nox inhibitors. In the present study, CellROX Green reagent was used to measure ROS levels in the corneas. To elucidate the role of Noxs in alkali burn-induced corneal oxidative stress, two common Nox inhibitors, DPI and Apo, were administered by eye drops. As shown in Fig. 2A, the ROS levels in the corneas were markedly increased after the alkali burns. By

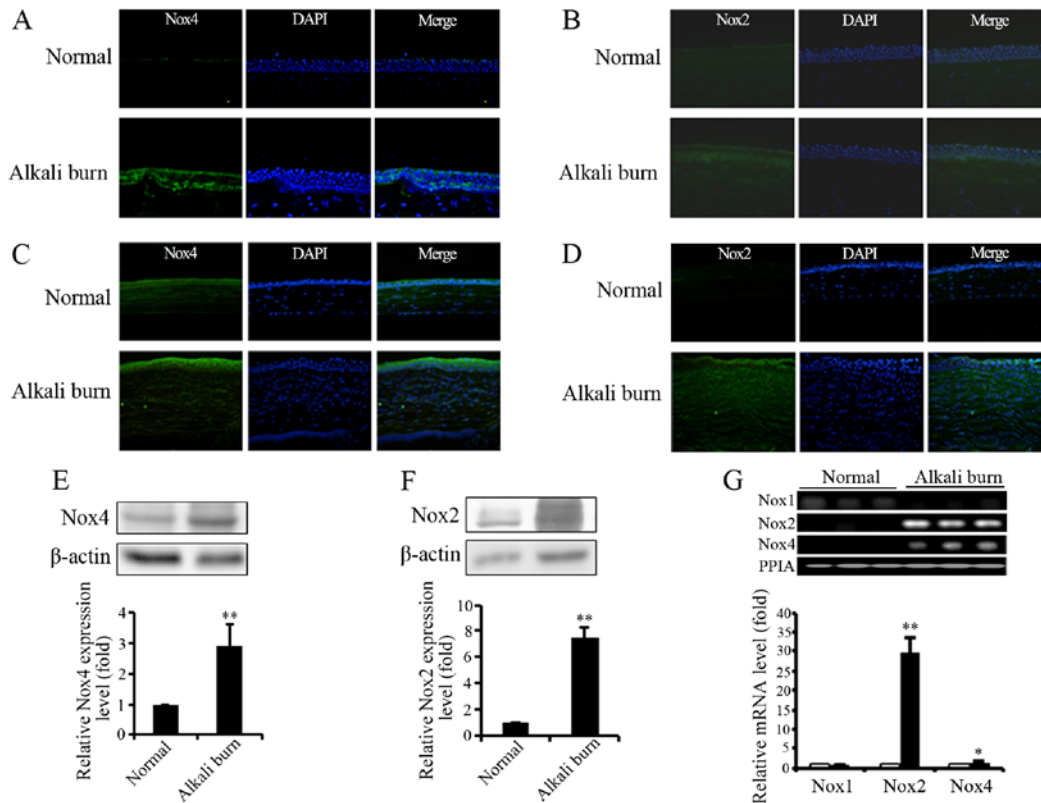


Figure 1. Expression of NADPH oxidase (Nox) isoforms in normal and alkali-burned corneas. (A) Expression of Nox4 was detected by immunofluorescence staining in human normal and alkali-burned corneas. Green indicates the fluorescence signal of Nox4, and blue indicates DAPI fluorescence (original magnification, x200). (B) Expression of Nox2 in human normal and alkali-burned corneas (original magnification, x200). (C) Expression of Nox4 and (D) Nox2 was detected by immunofluorescence staining in normal (upper panels) and alkali-burned (lower panels) corneas of mice (original magnification, x400). The protein level of (E) Nox4 and (F) Nox2 in mouse normal and alkali-burned corneas was examined by western blot analysis. β -actin was used as an endogenous control. The graphs indicate the normalized expression level of Nox4 or Nox2 in corneas. Quantification of Nox4 or Nox2 expression was indicated as the normalization of ratio of Nox4/ β -actin or Nox2/ β -actin in each sample to control. Data are presented as the means \pm SD of at least 3 independent experiments. ** $P < 0.01$. (G) RT-qPCR was applied to detect the gene transcription of Nox1, Nox2 and Nox4 in mouse corneas. PPIA was used as an endogenous control. Three independent experiments for each condition were carried out, * $P < 0.05$, ** $P < 0.01$ (compared with normal group). The upper panel shows the electrophoresis of the RT-qPCR product of Nox1, Nox2 and Nox4 on an agarose gel.

contrast, the Nox inhibitors, DPI or Apo, effectively reduced the levels of alkali burn-induced ROS in corneas. On the other hand, 3-NT was used to monitor peroxynitrite (ONOO⁻) formation. The appearance of 3-NT-containing proteins is a biomarker of oxidative stress, indicating the development of reactive nitrogen species (RNS) (28). Thus, the effect of Nox inhibitors on the level of 3-NT-containing proteins was further examined in the corneas. The results of immunofluorescence staining and western blot analysis revealed that the Nox inhibitors, DPI or Apo, significantly decreased the levels of 3-NT-containing proteins (Fig. 2B and C). Taken together, these results suggest that Noxs play an important role in alkali burn-induced oxidative stress in corneas.

Effect of Nox inhibitors on corneal inflammatory responses after alkali burns. It is well known that alkali burns trigger acute inflammation in the corneas, and ROS play an important role in the inflammatory response (9). As mentioned above, the expression of Noxs and oxidative stress were increased in corneas after alkali burns. Therefore, the effect of Nox inhibitors on the alkali burn-induced corneal inflammatory response was further examined. Firstly, the infiltration of inflammatory cells into the corneas was assessed using CD11b

immunofluorescence staining. As shown in Fig. 3A, alkali burns induced the marked infiltration of CD11b-positive inflammatory cells into the corneal stroma. The administration of DPI and Apo by eye drops markedly reduced the infiltration of CD11b-positive inflammatory cells. As shown in Fig. 3B, CD11b/Noxs double-immunofluorescence staining revealed the partial co-localization of CD11b with Nox2 and Nox4, which suggests the involvement of Nox2 and Nox4 in the infiltration of inflammatory cells in corneas after alkali burns. Moreover, RT-qPCR revealed that the mRNA levels of pro-inflammatory cytokines, namely IL-6, IL-1 β and TNF- α , were increased in the corneas on day 7 after alkali burns. The administration of DPI or Apo by eye drops significantly attenuated the alkali burn-induced increment of the mRNA levels of IL-6 and IL-1 β , but had no significant effect on the TNF- α levels (Fig. 3C). These results suggest that Noxs participate in the corneal inflammatory response through the regulation of inflammatory cell infiltration and the release of pro-inflammatory cytokines after alkali burns.

Inhibition of Noxs effectively attenuates alkali burn-induced CNV. Inflammation resulting from infection, aberrant immune responses, or chemical burns usually disrupts the balance

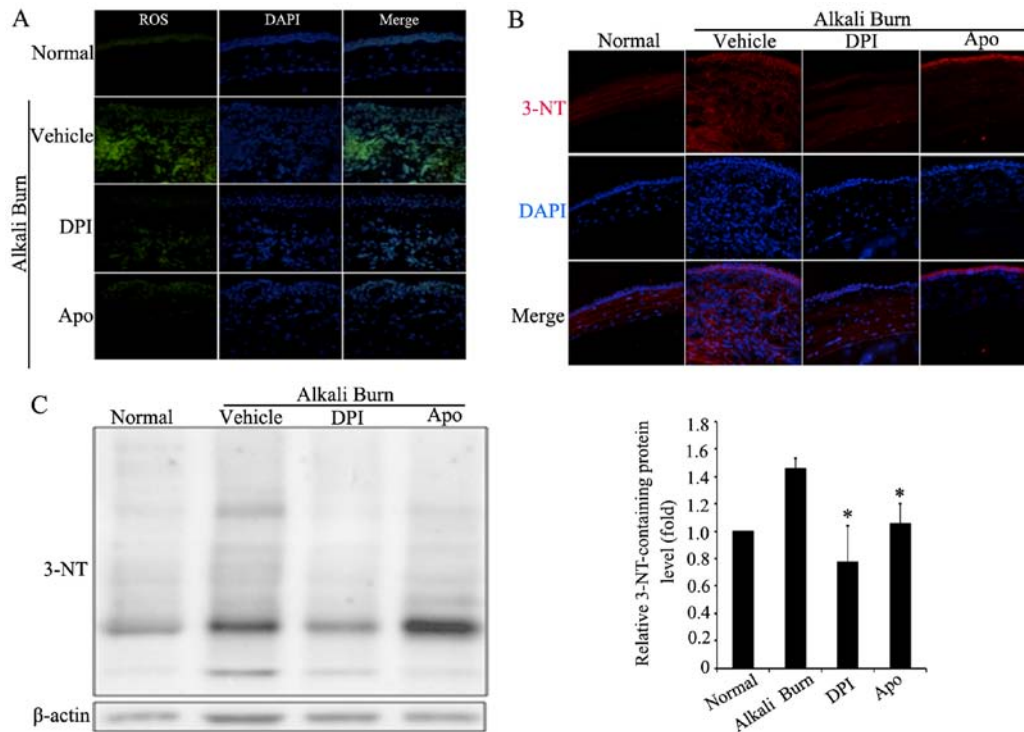


Figure 2. Diphenyleioidonium (DPI) and apocynin (Apo) effectively attenuate alkali burn-induced oxidative stress in corneas. (A) Effect of DPI and Apo on reactive oxygen species (ROS) production in corneas after alkali burns. ROS levels in corneas were examined using CellROX Green reagent. Green indicates the fluorescence signal of ROS, and blue indicates DAPI (original magnification, x400). (B) Immunofluorescence staining of 3-nitrotyrosine (3-NT)-containing proteins in the corneas. The specific antibody for 3-NT was used for immunofluorescence staining. Red indicates the immunofluorescence signal of 3-NT-containing proteins in corneal sections, and blue indicates DAPI (original magnification, x400). (C) 3-NT protein levels in the corneas. 3-NT-containing proteins in corneas were further examined by western blot analysis. β -actin was used as an endogenous control. The graph indicates the normalized expression level of 3-NT-containing proteins in the corneas. Quantification of 3-NT-containing protein levels was indicated as the normalization of ratio of 3-NT/ β -actin in each sample to normal. Data represent the means \pm SD of at least 3 independent experiments. * $P < 0.05$ vs. alkali burn group.

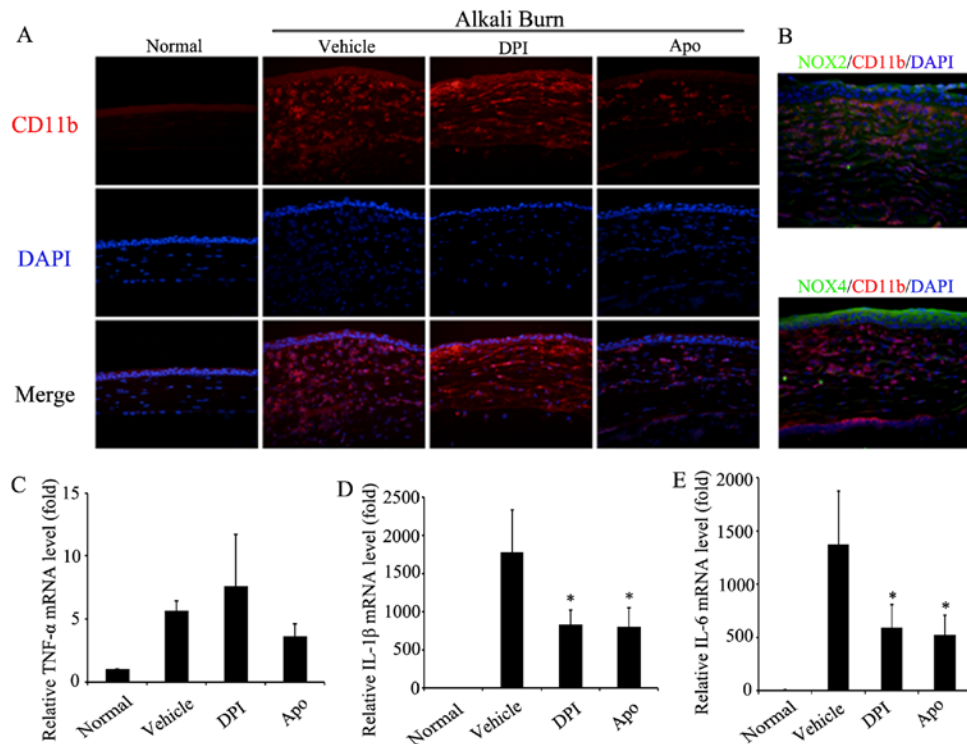


Figure 3. Inflammatory responses induced by alkali burns are suppressed by diphenyleioidonium (DPI) or apocynin (Apo). (A) Infiltration of inflammatory cells in corneas was detected by CD11b immunofluorescence staining. Green indicates the fluorescence signal of CD11b (original magnification, x400). (B) CD11b/NOXs double-immunofluorescence staining was used to examine the distribution of Nox2, Nox4 and the inflammatory marker CD11b. Red and green indicate the fluorescence signals of CD11b and Nox2/Nox4, respectively. (C-E) mRNA levels of pro-inflammatory factors in corneas. RT-qPCR was applied to detect the gene transcription of interleukin (IL)-1 β , IL-6 and tumor necrosis factor- α (TNF- α) in mouse corneas. Three independent experiments for each condition were carried out. * $P < 0.05$ vs. vehicle.

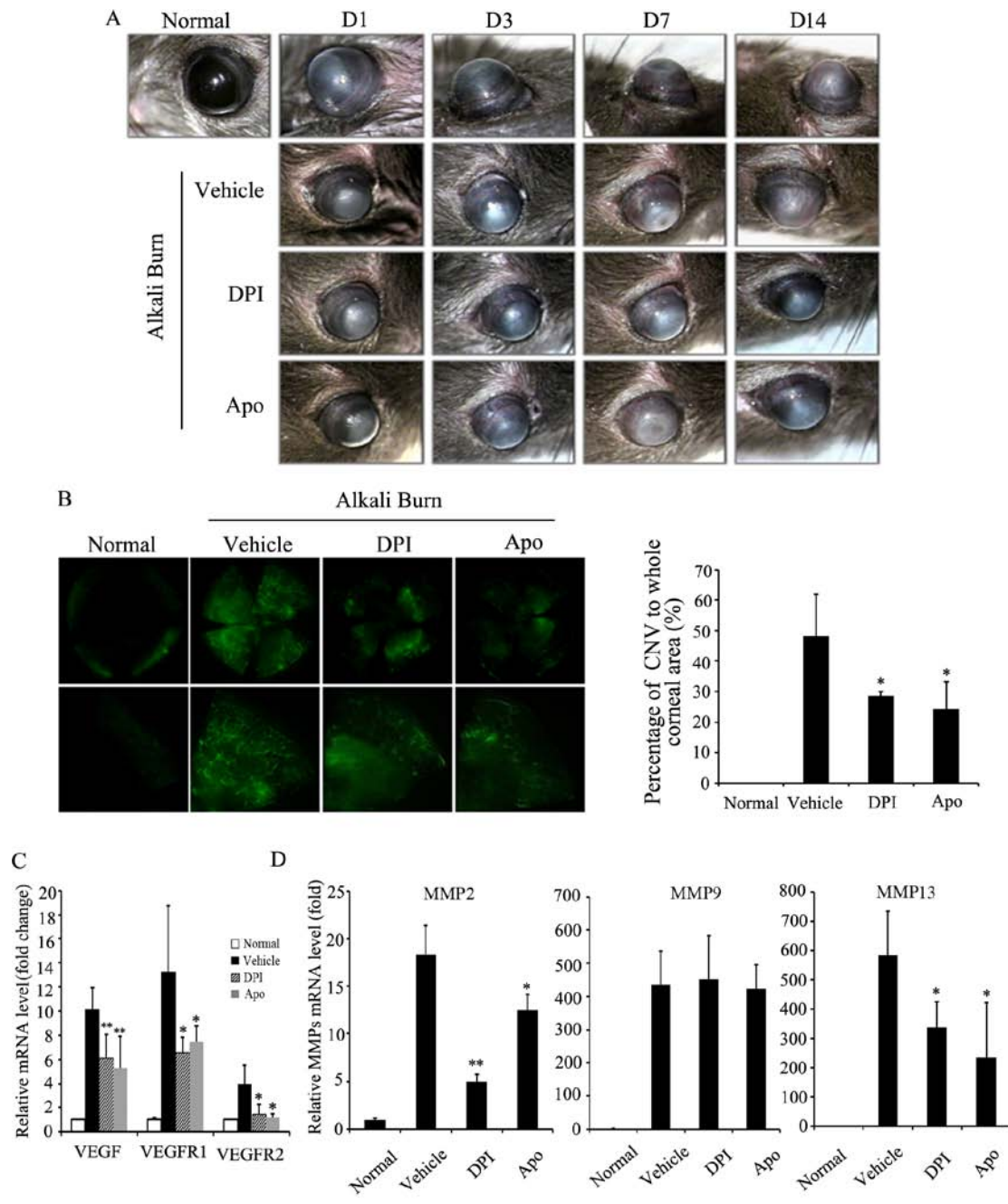


Figure 4. Effect of NADPH oxidase (NOX) inhibitors on corneal neovascularization after alkali burns. (A) Effect of diphenyleneiodonium (DPI) or apocynin (Apo) on the development of corneal neovascularization (CNV) after alkali burns. Corneal neovascularization was observed under a dissecting microscope and photographed over the time course of our study. Representative images of CNV at 1, 3, 7 and 14 days after alkali burns are shown. D, day. (B) Effect of DPI and Apo on CNV area after alkali burns. At 14 days after alkali burns, mice were sacrificed by a cardiac injection of FITC-dextran. The corneas were excised, and CNV was evaluated by FITC-dextran corneal angiography under fluorescence microscope (original magnification, x4). The graph shows quantification of CNV area in each condition. *P<0.05 vs. vehicle. (C and D) Effect of NOX inhibitors on the mRNA level of angiogenic factors in corneas after alkali burns. RT-qPCR was applied to evaluate the transcription of angiogenic factors, namely vascular endothelial growth factor (VEGF), VEGF receptor 1/2 (VEGFR1/2), matrix metalloproteinase (MMP)2, MMP9 and MMP13. Three independent experiments for each condition were carried out, *P<0.05, **P<0.01 vs. vehicle.

between angiogenic and anti-angiogenic factors and eventually triggers the development of CNV (29). In the present study, we monitored the development of CNV after alkali burns. As shown in Fig. 4A, CNV occurred on day 3, and it affected 50% of the whole area of the cornea on day 7, and on day 14, CNV coverage of the cornea reached maximum levels. DPI and Apo effectively suppressed the length and density of CNV on day 7 after alkali burns. To quantify the exact area of CNV

after alkali burns, FITC-dextran corneal angiography was conducted. As shown in Fig. 4B, both DPI and Apo significantly reduced the alkali burn-induced CNV area on day 14 after alkali burns. To further illustrate the role of Noxs in CNV, the mRNA levels of angiogenic factors, namely VEGF, VEGFR1 and VEGFR2 and MMPs in the corneas after alkali burns were measured by RT-qPCR. The results revealed that alkali burns markedly increased the mRNA levels of VEGF,

VEGFR1, VEGFR2, MMP2, MMP9 and MMP13 in the corneas. By contrast, administration of DPI or Apo by eye drops significantly reduced the alkali burn-induced transcription of these angiogenic factors, namely VEGF, VEGFR1/2, MMP2 and MMP13 (Fig. 4C and D). These results suggest that Noxs play an important role in chemical burn-induced CNV through the upregulation of angiogenic factors.

Discussion

Chemical burns remain an important cause of corneal damage. The extensive damage to corneas caused by chemical burns usually leads to visual impairment or loss of vision (30). It is well known that oxidative stress plays a crucial role in chemical burn-induced corneal damage, and oxidative stress is characterized by increased ROS production (31). As previously demonstrated, Noxs are an important source of ROS, which induces cellular oxidative stress, inflammation, tissue injuries and diseases (32). In the present study, we first examined the levels of Noxs in human and mouse corneas after alkali burns. The transcription and expression of Nox2 and Nox4 were significantly upregulated after alkali burns (Fig. 1). In addition, Nox2 and Nox4 were partially co-localized with CD11b-positive inflammatory cells in the corneal stroma (Fig. 3B). These results suggest that Nox2 and Nox4 are involved in the infiltration of inflammatory cells after alkali burns. Importantly, the topical administration of the Nox inhibitors, DPI or Apo, effectively attenuated alkali burn-induced oxidative stress and the infiltration of CD11b-positive inflammatory cells into the corneas (Figs. 2 and 3A). These results suggest the involvement of Noxs in alkali burn-induced oxidative stress and inflammatory responses in the cornea.

The inflammatory cells which infiltrate into the corneal stroma after chemical burns usually trigger the inflammatory responses through the release of inflammatory cytokines (1). In a previous study (33), the expression of IL-1, IL-6, IL-10 and TNF- α was detected in alkali-burned corneas. IL-1 and IL-6 levels were markedly upregulated at the early stages of alkali burn-induced injury, and the production peak occurred on days 3 and 7. By contrast, the production of IL-10 and TNF- α was not significantly increased after alkali burns. However, in the present study, we noted that the mRNA levels of IL-1 β , IL-6 and TNF- α were all elevated after alkali burns (Fig. 3C-E). The differences in results between the two studies are possibly due to the different evaluation methods used. Moreover, we noted that the administration of the Nox inhibitors, DPI or Apo, effectively reduced the increase in IL-1 β and IL-6 levels, but had no significant effect on TNF- α after alkali burns (Fig. 3C-E). These results suggest that the inhibition of Noxs effectively suppresses the release of inflammatory cytokines in corneas after alkali burns.

Under physiological conditions, the cornea is a transparent tissue. However, the inflammation induced by chemical burns, infection and aberrant immune responses often leads to pathological neovascularization in the corneas. CNV is one of the severe sequelae of alkali burns, and it greatly influences corneal transparency and results in permanent vision loss (1). In a previous study, it was reported that ROS derived from Nox upregulated angiogenesis-related factors and enhanced the migration and proliferation of endothelial cells (34). Consistent with this previous study, we found that alkali burns

markedly increased the area of CNV. The inhibition of Noxs by DPI or Apo significantly suppressed alkali burn-induced CNV (Fig. 4A and B). Moreover, DPI or Apo effectively attenuated the alkali burn-induced upregulation of the angiogenesis-related factors, VEGF, VEGFR1/2, MMP2 and MMP13 (Fig. 4C and D). These angiogenesis-related factors likely stimulate the proliferation, migration and tube formation of endothelial cells to promote neovascularization in corneas primarily through the VEGF receptors (35).

In conclusion, in the present study, we found that alkali burns upregulated the transcription and expression of Nox2 and Nox4 in corneas. The inhibition of Noxs by the administration of DPI or Apo effectively suppressed oxidative stress, inflammatory responses and neovascularization in the corneas after alkali burns. Although the role of Noxs in non-ophthalmological diseases has been previously described (36-39), our data demonstrate for the first time (to the best of our knowledge) that Noxs contribute to oxidative stress, inflammation and neovascularization in corneas after alkali burns. Thus, we suggest that the inhibition of Nox activity is a potential strategy for preventing corneal damage after chemical burns.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (grant no. 81300786) and the Jiangxi Science and Technology Department (grant no. 20132BAB205024) and the research grant from Jiangxi Education Department (grant no. GJJ13175), and supported partially by the National Natural Science Foundation of China (grant nos. 31360241 and 81472371).

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