Tetramethylpyrazine in a Murine Alkali-Burn Model Blocks NFκB/NRF-1/CXCR4-Signaling-Induced Corneal Neovascularization

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Corneal neovascularization (CNV) obscures the corneal transparency essential for optimal vision.1 New blood vessels usually occur in response to alkali burns, keratitis, trauma, and keratoplasty, which can all damage vision and even cause blindness.2 CNV arises from the pre-existing vasculature that surrounds the cornea. During neovascularization, new vessels invade and grow in the normally avascular cornea, severely impairing vision.3,4 CNV is fostered by a variety of angiogenic factors, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor, and chemokine receptor 4 (CXCR4).5-10 Our previous studies showed that tetramethylpyrazine (TMP) inhibits CNV by downregulating CXCR4 expression.10

Chuanxiong is an herb that, for over 2000 years, has been used in traditional Chinese medicine to treat a variety of diseases. The active component, TMP, shows strong antiangiogenic properties, and neuroprotective and antitumor effects, both in vitro and in vivo.11-15 This herbal supplement remarkably promotes microcirculation and improves blood flow by attenuating thrombus formation, decreasing blood viscosity, dissipating blood stasis, and dilating blood vessels, and thus is an effective therapy for numerous cardiovascular and cerebrovascular diseases.10 TMP was recently proven to inhibit neovascularization in ophthalmologic diseases, with mild side effects.16 Early investigations revealed TMP decreases CNV and inhibits neovascularization in ischemic retinas, but the mechanisms of TMP’s antiangiogenic effects have remained unclear.

Our previous study suggested TMP might attenuate CNV by targeting CXCR4, which is linked to vascular formation. Although CXCR4 expression is regulated by various transcriptional factors in different processes, those targeted by TMP remained unknown.

CXCR4 is a 7-transmembrane domain, G-protein-coupled receptor, expressed in a variety of cells, including vascular endothelial cells, neurons, and cancer cells.17-19 Its normal functions appear to be diverted, causing it to drive neovascularization of various diseases, such as pathologic neovascularization, myocardial infarction, cancer, and inflammation.20-22 Under pathologic conditions, CXCR4 also boosts neovascularization in tissues, including the cornea and the retina. In diverse settings, various transcription factors control CXCR4 expres-
ion, including nuclear factor kappa B (NFkB), nuclear respiratory factor-1 (NRF-1), forkhead box C1 (FoxC1), and yin yang 1 (YY1). The transcription factor YY1 was reported to bind the CXCR4 promoter during breast cancer progression and metastasis and during human herpes virus 6 infection; and YY1, along with CXCR4, intervenes in the pathogenesis of osteosarcoma on cell invasiveness. In addition, FoxC1 activation of CXCR4 expression controls mesenchyme differentiation and chemotactic motility of endothelial cells.

Previously, we showed NRF-1 regulates CXCR4 transcription in the rat retina. Because NRF-1 reportedly activates the CXCR4 promoter during entry of the human immunodeficiency virus type 1, NFkB is upregulated in retinal vein occlusion and patients with diabetic retinopathy; these two proteins seemed like likely potential targets of therapeutic TMP treatment for CNV.

Our studies in vivo, in alkali burn-injured mice, confirm TMP inhibits CNV by downregulating expression of CXCR4, NFkB, and NRF-1. Moreover, our chromatin immunoprecipitation (ChIP) and genetic interference assays show NRF-1 binds the CXCR4 promoter directly, and TMP treatment decreased this binding efficiency in vitro. Additionally, NFkB interacted with CXCR4 indirectly by regulating NRF-1 expression and repressing downstream-target gene expression. Thus, the use of TMP to target NRF-1 and NFkB may have therapeutic potential as a treatment for CNV disorders.

**Materials and Methods**

**Cell Culture**

The human umbilical vein endothelial cell (HUVEC) line was obtained from the ATCC Collection (Manassas, VA, USA) and grown in Dulbecco’s modified Eagle’s medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum in humidified conditions of 5% carbon dioxide at 37°C. The HUVEC line used in experiments was the same batch at different passages. TMP applied to cells was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA) and dissolved in a component solvent (saline:dimethyl sulfoxide 1:1) to reach a concentration of 200 μmol/L. The same amount of component solvent was used as a control in all cell experiments accordingly.

**Animals and Model of Corneal Alkali Burn**

A total of 80 C57BL/6j mice aged 6 to 8 weeks were purchased from the Guangdong Provincial Center for Animal Research (Guangzhou, China). Their right eyes were selected for experimentation. After anesthetized with an intraperitoneal injection of 4% paraformaldehyde for 15 minutes, and subsequently treated with 10% goat serum for 30 minutes. Cells were incubated with primary antibodies against CXCR4 (1:100; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), NRF-1 (1:100; Abcam, Cambridge, MA, USA), and NFkB (Boster, Wuhan, China) overnight at 4°C. Secondary anti-mouse or anti-goat antibody (1:500 dilution, Cell Signaling Technology, Danvers, MA, USA) was added at room temperature, and DAPI was used to stain nuclei. Photomicrographs were captured by fluorescence system.

**Real-Time RT-PCR**

Expression of CXCR4, NRF-1, NFkB, YY1, and FoxC1 was measured after TMP treatment by real-time PCR analyses by using the SYBR Green system (Takara, Tokyo, Japan). The following primer pairs were used: for β-actin, 5′-AGTCACACCTATGGGCAAGG-3′ (sense) and 5′-ACGGATGTCAAACGCTACACCT-3′ (antisense); for CXCR4, 5′-TTCTCTATCCGCTTCGCTC-3′ (sense) and 5′-GTCTTTCTGCGAGATCCATC-3′ (antisense); for NRF-1, 5′-AGCACGTGGTGGACCCAC-3′ (sense) and 5′-TGTAGGGTGTTGAGGGT-3′ (antisense); for NFkB, 5′-GGAAAAGAAAGCGCGAGGCGACTGCACCCCT-3′ (sense) and 5′-TGAGAAGAGGCATTGAAATGACCTGGAACAGGGAGGGT-3′ (antisense); and for FoxC1, 5′-AGTTGAGGACCATCTGCAACCCAC-3′ (sense) and 5′-AGTCACCTGGAACCCAC-3′ (antisense).
TMP Blocks NFkB/NRF-1/CXCR4 Axis

Hyde-na-phosphate dehydrogenase (GAPDH; 1:5000; Protein-tech, Rosemont, IL, USA) served as a loading control.

**Cell Viability Assays by MTT**

The viability of HUVECs was detected using the MTT assay 24 and 48 hours after TMP treatment. With 100 μl of dimethyl sulfoxide added to each well in 96-well plates, cells were incubated at 37°C for 4 hours. Absorbance was measured at 490 nm with a fluorescence plate reader (Power Wave XS; BioTek, Winooski, VT, USA). Cell viability was determined using the optical density ratio of a treated culture relative to an untreated control.

**Chromatin Immunoprecipitation (ChIP) Assay**

After treated with 200 μm/L TMP, HUVECs were subjected to a ChIP assay, according to the manufacturer's instructions using the ChIP assay kit (Upstate Cell Signaling Solutions, Lake Placid, NY, USA). The antibodies NRF-1 (Abcam), NFκB (Cell Signaling Technology), or normal IgG (Sigma-Aldrich Corp.) were used. DNA was purified by phenol/chloroform extraction and ethanol precipitation and amplified by PCR by using primers spanning the activated transcription factor site of the CXCR4 promoter (forward, 5'-ACAGAGAGACGCGTTCCTAG-3'; reverse, 5'-AGCCCGAGGAGCTCTCCTG-3') and NRF-1 promoter (forward, 5'-GGTCCAGGAATCCTCAAACA-3'; reverse, 5'-CAGGTTGCTAGAAGTGGG-5').

**Plasmid Construction**

The plasmid pEPI-NRF-1 was derived from pEPI-GFP (generously provided by H.J. Lipps); NRF-1 cDNA was inserted in pEPI-GFP by restriction-digested sites EcoRI and BamHI. The plasmid pEV-p105-NFκB was bought from Addgene (Cambridge, MA, USA).

**Ethics Statement**

All studies complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All procedures were approved and monitored by the Institutional Animal Care and Use Committee of Zhongshan Ophthalmic Center (permit number, SYXK [YUE] 2015-135). Animal care staff and veterinary personnel monitored animal health.

**Statistical Analysis**

All experiments were performed in triplicates or more. Data are expressed as means ± SE. The two-tailed Student’s t-test was used to calculate the differences between mean values. All calculations and statistic tests were analyzed using GraphPad Prism 6 for Mac version 4.02 (GraphPad Software, San Diego, CA, USA) or Microsoft Excel 2003 (Microsoft, Redmond, WA, USA). A P value no more than 0.05 was considered significant.

**RESULTS**

**CNV Induced by Alkali Burn Was Inhibited by TMP Treatment In Vivo**

Because CNV peaks 2 weeks after injury, we measured the area of neovascularization at that time. At day 14, mice subjected to TMP treatment or PBS treatment were anesthetized and photographed by slit lamp (Fig. 1B, 1C), showing neovascularization (Fig. 1B) invaded the central cornea, staining robustly in the PBS-treated group. Comparatively, the TMP-treated group developed only modest neovascularization that was mainly restricted to the cornea periphery (Fig. 1C). According to corneal flat-mounts stained with anti-CD31-fluorescent antibody, the area of neovascularization in the TMP-treated group was significantly smaller than that in the injury group (Fig. 1E, 1F). Staining was examined under magnification (Fig. 1H, 1I), showing neovascularization in the injury group was denser and longer than in the TMP-treated group. In the TMP-treated group, the relative degree of CNV was significantly lower than that of the injury group (43.29% and 23.70%, respectively) (Fig. 1J).

The murine corneas were examined with a surgical microscope and scored according to the observed degree of edema, corneal opacity, area of neovascularization, and vessel size, with scores reflecting the severity of injury. By these criteria, the average score of the TMP-treated group was significantly lower than the PBS-treated group (8.853 and 4.454, respectively) (Fig. 1K).

Real-time PCR showed injury-triggered pathologic CXCR4 overexpression (12.41 ± 7.87-fold) in the mouse cornea that could be repressed by TMP treatment (5.26 ± 2.07-fold), strongly suggesting that TMP attenuates CNV by inhibiting CXCR4 expression (Fig. 1L).

**The Expression Levels of the NRF-1 and NFκB Transcription Factors Correlate With CXCR4 Expression**

Depending on the biologic setting, various genes can regulate the CXCR4 promoter, including NRF-1, NFκB, FoxC1, and YY1, but it is not clear which factors might be the target(s) of TMP-mediated CNV inhibition. To determine this, we measured the expression changes of NRF-1, NFκB, FoxC1, and YY1 at day 3 after injury. According to RT-PCR results, expression of both NRF-1 and NFκB was stimulated by injury but reduced by TMP treatment—changes that mirrored the trend of CXCR4 expression. As shown in Figure 2A, TMP treatment after injury downregulated NRF-1 expression compared to the untreated control (Fig. 2B; control, 1-fold; the injury group, 1.83 ± 0.62-fold; the TMP-treated group, 0.71 ± 0.23-fold). NFκB expression ran parallel to the trend of NRF-1 expression: injury upregulated NFκB but this could be ameliorated by TMP treatment (Fig. 2B; control, 1-fold; the injury group, 2.69 ± 1.60-fold; the TMP-treated group, 0.89 ± 0.50-fold). In contrast, expression of FoxC1 and YY1 declined after injury, and the PBS-treated and TMP-treated groups showed no statistic difference in expression of FoxC1 and YY1 (Fig. 2C, 2D). These results show TMP effectively downregulates CXCR4, NRF-1, and NFκB expression in murine corneas, suggesting NRF-1 and NFκB might be the transcription factors targeted by TMP during neovascularization inhibition.

**CXCR4, NRF-1, and NFκB Expression Decreases In Vivo After TMP Treatment**

Immunofluorescence staining at day 14 postinjury showed the corneas of the TMP-treated group were thinner than in the injury group, indicating significantly milder edema and stromal inflammation with TMP treatment. Because VEGF is well-known to induce neovascularization in vivo, fluorescence intensity was compared among the 3 groups, showing strong VEGF expression in the corneal epithelium, especially in the cytoplasm after injury (Fig. 3A). Treatment with TMP eye drops significantly reduced the intensity of VEGF fluorescence. The staining intensity of CXCR4 was also notably reduced in the TMP-treated group (Fig. 3B). To explore what upstream changes might influence CXCR4 expression, murine corneas were also fluorescently stained for NRF-1. As shown in Figure...
FIGURE 1. TMP inhibits CNV in an alkali-burn-induced murine model. (A–C) Regression of CNV, especially in the area along the central visual axis, was observed in the TMP-treated group. Photos were taken by slit lamp at day 14. (D, G) Corneal flat mounts stained with the vascular endothelial cell marker PECAM-1 antibody (green) at various magnifications in uninjured group. (E, H) Corneal flat mounts stained with PECAM-1 antibody at various magnifications show an extensive network of new blood vessels around corneal limbus in an injured subject treated with PBS after alkali injury. (F, I) Corneal flat-mount staining indicates neovascularization is severely suppressed in TMP-treated eyes at 14 days. (J) Quantification of the neovascularized area from each group of mice by Image J. (K) Clinical assessment scores based on the degree of corneal opacity, vessel size, edema, and others, with the help of digital camera at day 14. The average scores of the TMP-treated group were significantly lower than the scores of the PBS-treated group. (L) CXCR4 mRNA expression in the uninjured group, the injured group treated with PBS, and the injured group treated with TMP, as detected by RT-PCR at day 3. CXCR4 expression increased after injury, as compared to the uninjured mice. CXCR4 expression was inhibited by TMP treatment at day 3. \( n = 10 \) /group; * \( P < 0.05 \), ** \( P < 0.01 \).

FIGURE 2. Effects of TMP on mRNA production of CXCR4-related genes in murine corneas after alkali burn injury, as detected by real-time RT-PCR. (A) Relative mRNA expression of NRF-1 detected by real-time RT-PCR in mice cornea. (B) Relative mRNA expression of N\( \text{Fk} \)B detected by real-time RT-PCR in murine corneas. (C, D) Real-time RT-PCR in murine corneas shows relative mRNA expression of FoxC1 and YY1. The groups without injury (Con), the group treated with PBS after injury (Injury), and the group treated with TMP were observed at day 3. \( n = 9 \) /group; * \( P < 0.05 \).
After alkali injury, nuclei in the corneal epithelium stained positive for NFκB, indicating that NFκB had translocated from the cytoplasm to the nuclei. With treatment by TMP eye drops, the staining intensity of NFκB was markedly weaker (Fig. 3D). Thus, treatment of TMP attenuated corneal edema and inflammation and imposed an inhibitory effect on the expression of VEGF, CXCR4, NRF-1, and NFκB.
Treatment Targets the Transcription Factors NRF-1 and NFκB In Vitro

To confirm the above results, expression of these genes was tracked in HUVECs with or without 200 μmol/L TMP treatment. Immunofluorescence staining showed that CXCR4 and NFκB were normally located in the cytoplasm, with NRF-1 mainly staining in nuclei (Fig. 4A). At different time points after TMP treatment, HUVECs were extracted for real-time PCR and Western blot assays, which showed that CXCR4 mRNA was downregulated in a time-dependent manner after TMP treatment (Fig. 4B; for CXCR4: control [con], 1; 24 hours, 81.32% ± 9.75%; 48 hours, 56.25% ± 4.04%; for NRF-1: con, 1; 24 hours, 81.91% ± 11.22%; 48 hours, 77.26% ± 2.15%; for NFκB: con, 1; 24 hours, 84.43% ± 8.28%; 48 hours, 68.78% ± 3.37%). Treated cells were extracted for Western blot analysis, at the same time point, and the relative intensity of the bands (normalized to GAPDH) was quantified by densitometry and compared. Consistently, a decrease was observed at the posttranscriptional level (Fig. 4C, 4D; for CXCR4: con, 1; 24 hours, 70.92% ± 0.43%; 48 hours, 38.52% ± 0.63%; for NRF-1: con, 1; 24 hours, 91.58% ± 2.50%; 48 hours, 67.16% ± 3.72%; for NFκB: con, 1; 24 hours, 93.11% ± 3.26%; 48 hours, 54.98 ± 0.13%). These results supported the idea that TMP inhibited neovascularization by downregulating CXCR4, likely via inhibition of NRF-1 and NFκB. According to results of the MTT assay (Fig. 4E), with the presence of 200 μmol/L TMP, the survival rate of HUVECs was reduced to 95.77% ± 1.03% at 24 hours and 93.34% ± 1.38% at 48 hours. Thus, TMP is clinically safe.

The Effects of NFκB and NRF-1 on CXCR4 Transcription In Vitro Are Not Synergistic

To confirm that NRF-1 and NFκB regulate the CXCR4 promoter, the transcription factors were expressed in HUVECs from recombinant transfection vectors (pEPI-NRF-1 and pEV-p105-NFκB). Two days after transfection, cells were collected and expression of NRF-1, NFκB, and CXCR4 was evaluated by Western blotting assays, with GAPDH as a loading control. Here, in HUVECs overexpressing the recombinant NRF-1, CXCR4 expression was elevated (Fig. 5A; for NRF-1: con, 1-fold; +pEPI-NRF-1, 1.93 ± 0.12-fold; for CXCR4: con, 1-fold; +pEPI-NRF-1, 2.14 ± 0.41-fold). Similarly, the CXCR4 expression level increased with overexpression of NFκB (Fig. 5C; for NFκB: con, 1-fold; +pEV-p105-NFκB, 1.15 ± 0.06-fold; for CXCR4: con, 1-fold; +pEPI-NFκB, 1.78 ± 0.36-fold). Consistent with our experimental results in vitro, overexpression of both transcription factors had a not-quite-additive effect on CXCR4 expression, with the fold elevation in CXCR4 transcription in the presence of both (at 2.69 ± 0.78-fold elevation) being less than the sum of the fold elevation of NRF-1 (2.14 ± 0.41-fold) and NFκB plasmid (1.78 ± 0.36-fold) (Fig. 5E, 5F; for NRF-1: con, 1-fold; +pEPI-NRF-1 and pEV-p105-NFκB, 2.10 ± 0.10-fold; for NFκB: con, 1-fold; +pEPI-NFκB and pEV-p105-NFκB, 2.11 ± 0.02-fold; for CXCR4: +vector, 1-fold; +pEPI-NRF-1 and pEV-p105-NFκB, 2.69 ± 0.78-fold).

NRF-1 Directly Activates CXCR4 Transcription, and NFκB Increases NRF-1 Expression In Vitro

The promoters bound by NRF-1 and NFκB in HUVECs were analyzed by ChIP at 48 hours after TMP treatment. As shown in Figure 6A, DNA was sonicated to fragments of ~0.4 kb in length. After precipitated with rabbit antibodies to normal rabbit IgG (or NFκB and NRF-1), DNA was extracted for PCR or real-time PCR amplification using CXCR4 primers of 145 bp. In both control and TMP-treated HUVECs, the NRF-1 antibody immunoprecipitated DNA from which our CXCR4-promoter-specific primers robustly amplified a 145-bp product (Fig. 6B), reflecting that NRF-1 binds the CXCR4 promoter directly. Real-time PCR compared CXCR4 expression in the control and TMP-treated groups, showing that the effect of NRF-1 on CXCR4 mRNA expression could be repressed by TMP treatment (Fig. 6C; con, 1; the TMP treated group, 73.67% ± 9.49%). Surprisingly, however, ChIP analysis suggested NFκB
does not bind the CXCR4 promoter (Fig. 6D), implicating NRF-1, but not NFκB, as a direct transcriptional regulator of the CXCR4 promoter.

Because our results suggest that NFκB might influence CXCR4 expression indirectly, by regulating the NRF-1 promoter, we analyzed the effect of overexpressed, recombinant NFκB on the NRF-1 promoter (Fig. 6E, 6F). As expected, recombinant NFκB overexpressed from a transfection vector induced stimulated NRF-1 expression, implicating NFκB as a regulator of NRF-1 in endothelial cells.

**FIGURE 5.** Western blot assays show that NRF-1 and NFκB promote transcription of CXCR4 in HUVECs. (A) Western blotting shows transfection of NRF-1 plasmid increased the expression level of CXCR4 in HUVECs. (B) Relative fold expression change of NRF-1 and CXCR4 after transfection of NRF-1 plasmid. (C) Transfection of exogenous NFκB elevated CXCR4 expression in HUVECs, as detected by Western blotting. (D) Relative fold change expression of NFκB and CXCR4 after transfection of the NFκB plasmid. (E) Cotransfection of NRF-1 and NFκB plasmids into HUVECs upregulated the expression of NRF-1, NFκB, and CXCR4. (F) Relative fold change in the expression of NRF-1, NFκB, and CXCR4 after cotransfection. 

**FIGURE 6.** (A) Sonicated DNA isolated from HUVECs and treated with formaldehyde to crosslink endogenous proteins to DNA. (B, D) ChIP assay with NRF-1 or NFκB antibodies and a control, normal rabbit IgG. Immunoprecipitations were PCR amplified to yield a 145-bp fragment of the CXCR4 promoter spanning the activated transcription factor sites. (C) The samples from NRF-1 antibody ChIP assays were probed by real-time RT-PCR to analyze the expression of CXCR4 in the control and TMP-treated groups. (E) The expression of NRF-1 increased after transfection of the NFκB promoter in HUVECs. (F) Relative fold change expression of NRF-1 after transfection of the NFκB plasmid. 

$^* P < 0.05$, $^{**} P < 0.01$. 

n = 3;
DISCUSSION

In our mouse alkali-burn model of corneal injury, therapeutically applied TMP remarkably attenuated neovascularization by downregulating CXCR4 expression. Similarly, in our transcription assays in transfected cells, TMP blocked upregulation of NRF-1, NFκB, and CXCR4. Thus, our study suggests a mechanism for TMP’s effect on CXCR4: TMP downregulates NFκB, which in turn prevents NFκB from pathologically overstimulating the NRF-1 to drive CXCR4 expression—a novel mechanism in which the NFκB/NRF-1/CXCR4 signaling pathway links TMP to inhibited neovascularization.

CXCR4 plays an important role in neovascularization and is regulated by various transcription factors in different processes or cell types. For example, YY1 binds the CXCR4 promoter during cancer invasiveness, progression, and metastasis. FoxC1 directly induces CXCR4 expression by activating its promoter in endothelial cells, and NRF-1 might be of critical importance in the proliferation of vascular smooth muscle cells (VSMCs) and during mitochondrial biogenesis in human vein endothelial cells. In the latter example, NFκB could promote VSMC proliferation and necroinflammation, contributing to atherogenesis and progression of atherosclerotic plaque after arterial injury. Such vascular inflammation and neovascularization might be attenuated by suppressing NFκB.

In our study, CXCR4, NRF-1, and NFκB were prominently expressed in the epithelial layer of the murine cornea (Fig. 5), and all of them were abnormally activated in parallel with alkali injury in vivo. TMP significantly downregulated the expression of CXCR4, NRF-1, and NFκB in parallel in CNV, making these transcription factors likely targets of therapeutic TMP for corneal injury.

Our data implicate NFκB as the target of therapeutic TMP, suggesting its effect on CXCR4 is indirect. TMP also protects endothelial cells from oxidative stress by downregulating NRF-1 expression. This is likely relevant because repression of NFκB expression by TMP is anti-inflammatory in HUVECs and inhibits VSMC proliferation. Although NFκB does not directly bind the NRF-1 promoter (Fig. S1), supporting reports showed NFκB regulates the NRF-1 promoter, affecting downstream, target-gene expression. By extension, these data support the idea that TMP inhibits CNV via the NFκB/NRF-1/CXCR4 signaling pathway, which might be one of several, complex signaling pathways that respond to TMP in CNV. Going forward, other TMP targets will likely be uncovered by future studies, further supporting the modern clinical use of this ancient healing agent.

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