A New Recombinant PACAP-Derived Peptide Efficiently Promotes Corneal Wound Repairing and Lacrimal Secretion

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PURPOSE. A new recombinant pituitary adenylate cyclase-activating polypeptide (PACAP)-derived peptide, MPAPO, which has higher stability and PAC1-specific potency, was generated. The actions of MPAPO on corneal wound repairing and lacrimal secretion were examined.

METHODS. MPAPO was prepared and identified by gene recombination, high-performance liquid chromatography (HPLC), and electrospray ionization mass spectrometry (ESI-MS). Stability assay was performed by HPLC-ESI-MS. PAC1-specific binding and potency assays were performed using PAC1-CHO cells. C57BL/6 mice and Japanese white rabbits were respectively used to analyze the effects of MPAPO on corneal wound repairing and lacrimal fluid secretion. Tetrazolium-based colorimetric assay (MTT), immunofluorescence, gene microarrays, and Western blot assay were performed to measure the effects of MPAPO on corneal epithelial cell proliferation, synapse growth, and gene differential expression of trigeminal ganglion cells.

RESULTS. As compared with the wild PACAP, the in vitro stability and PAC1-specific potency of MPAPO with four mutations (M17L, L27K and M, K, respectively, added to the N- and C-terminus) were increased approximately 31- and 2-fold, respectively. MPAPO can significantly promote the proliferation of mouse corneal epithelium cells and the synapse growth of trigeminal ganglion cells. In experimental animals, MPAPO performed a complete corneal epithelial wound closure in 30 hours and significantly inhibited corneal neovascularization, and the effects were obviously stronger than for wild PACAP and recombinant bovine (rb)-bFGF (an anti-corneal wound drug). Furthermore, MPAPO can increase the lacrimal secretion, which may efficiently improve dry eye.

CONCLUSIONS. MPAPO may represent a promising external therapeutic peptide for corneal wound repairing or dry eye.

Keywords: gene recombination, pituitary adenylate cyclase activating polypeptide (PACAP)-derived peptide, PAC1 receptor, high stability, corneal wound repairing

Pituitary adenylate cyclase-activating polypeptide (PACAP) is one of the important neuropeptides and a new member in the secretin/glucagon/vasoactive intestinal peptide (VIP) family.1 Pituitary adenylate cyclase-activating polypeptide is present in two forms, PACAP38 and PACAP27 in vivo, and has 60% homology with VIP, but the capacity of PACAP for stimulating brain pituicytes to generate adenylate cyclase is 1000-fold that of VIP.2 The biological actions of PACAP are mediated through three G protein-coupled receptors, namely, PAC1, VPAC1, and VPAC2. PAC1 is the specific 7-transmembrane receptor and exhibits high affinity for PACAP, but much lower affinity for VIP. VPAC1 and VPAC2 receptors display similar affinity for PACAP and VIP.3 In mammals, PAC1 and VPAC receptors are expressed in the nervous, gastrointestinal, reproductive, and immune systems. However, PAC1 is mainly expressed in endocrine glands, such as the pituitary and adrenal, in the eye, and in the peripheral nervous system (PNS). In the PNS, especially in many ganglia and axons, PAC1 receptor distribution is significantly higher than that of VPAC receptors.4,5

A growing body of evidence suggests that PACAP can efficiently promote traumatic brain or nerve injury repair and nerve synapse growth and modulate the function of the immune system.6,7 Pituitary adenylate cyclase-activating polypeptide was strongly upregulated in injured neurons, including motor, sympathetic, and sensory neurons.8,9 Pituitary adenylate cyclase-activating polypeptide-deficient (knockout, KO) mice exhibited delayed axonal regeneration in a facial nerve crush model, and PACAP administration in vivo can attenuate axon degeneration in central nervous system (CNS) injury models.10,11 In PACAP KO mice, exogenous PACAP treatment appeared to reduce neuronal damage resulting from a lack of endogenous PACAP expression and afforded a degree of extra protection, equivalent to that observed with the wild mice.12 Pituitary adenylate cyclase-activating polypeptide and PAC1 are highly expressed in the embryonic proliferative zone and neural tubes, indicating that PACAP has a regulation effect on the development of neurons and glial cell precursors; in addition, in tissue culture, PACAP played key roles in the origin, development, and proliferation of the nerve cells.13 The PACAP-PAC1 autocrine system promotes proliferation in neural

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progenitor cells via Ca\(^2+\)-dependent signaling pathways through PAC1.\(^{14}\) Therefore, PACAP or a PAC1-specific agonist has been proposed as a potential agent for injury repair.

However, the therapeutic application of PACAP or some PAC1-specific agonist has been hampered by their poor stability and limited bioavailability in vivo.\(^{15,16}\) Particularly, PACAP is susceptible to protease degradation by dipeptidyl peptidase-4 (DPP4), which causes loss of the first two N-terminal amino acids (H-S) required for receptor activation.\(^{17}\)

Furthermore, the 17th amino acid is methionine (M) and prone to oxidation, thereby leading to the destabilization and low bioavailability of this peptide. Many approaches have been explored to improve its pharmacokinetics, including PEGylation, coadministration of peptide-degrading enzyme inhibitors, and various delivery systems.\(^{18-20}\) However, success in these strategies has been limited by the propensity of the large polymers to interfere with peptide function. In the current study, we have developed the novel recombinant DPP4-resistant peptide MPAPO with 29 amino acids by gene recombinant technique. Compared with PACAP27, a methionine (M) and a lycine (K) was respectively added at the N- and C-terminus of MPAPO; in addition, we have introduced two mutations (M17L, L27K) into the peptide MPAPO, so as to increase its stability by eliminating oxidation and DPP4 degradation and also improve its potency to activate PAC1 receptor. Thus the in vitro stability of recombinant MPAPO was approximately 31-fold higher than that of PACAP and VIP.

MPAPO can quickly perform mouse corneal epithelial wound repair due to its high stability and bioavailability. Furthermore, MPAPO exhibits potent effects in increasing lacrimal fluid secretion and inhibiting corneal neovascularization, which is one significant side effect caused by some growth factor therapeutics.

**Preparation and Identification of the Recombinant Peptide MPAPO**

The MPAPO gene was designed according to the bias of *Escherichia coli* for the codons to ensure its high expression. The gene was synthesized and amplified in two steps as described previously\(^{22}\) using three oligonucleotide primers: F1: 5′-GGT GGT CATA CTG AGC GAT GGC ATT TTG ACC GAT AGC TAT AGC-3′, containing an Ndel site (underlined); F2: 5′-TTT TTT CAC CGC CAG CTC TTT GCG ATA GCG GCT ATA GCT ATC GGT GGT-3′; F3: 5′-CCACCATGCTCTCCGCA TTT TTT-3′, containing a SapI site (underlined); GGTGGT at the 5′ end of F1 and CCACCA at the 5′ end of F3 are the protecting bases. After polymerase chain reaction (PCR), the products were purified by the PCR cleanup kit (Qiagen, Hilden, Germany) and digested with Ndel and SapI; the DNA fragment was directly ligated to pKYB-MCS vector (NEB, Ipswich, MA, USA) to yield the expression plasmid pKYB-MPAPO. MPAPO gene in plasmid pKYB-MPAPO was verified by DNA sequencing. The vector pKYB-MPAPO was transformed into *E. coli* ER2566 and the fusion proteins were expressed and purified by the optimized procedure previously described.\(^{23}\) After chloram hydrin (NEB) affinity chromatography purification for the cell lysate, MPAPO was purified and prepared by reverse-phase high-performance liquid chromatography (HPLC) using 4.6-×150-mm 300 SB-C18 Sep-Pak column (Agilent Technologies, Beijing, China) through gradient elution of acetonitrile from 3% to 60% for 20 minutes at 0.5 mL/min. The eluate containing MPAPO was dried by lyophilization. Prepared MPAPO was identified by tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (tricine-SDS-PAGE) and 4000 Q TRAP electrospray ionization mass spectrometry (ESI-MS) (Applied Biosystems, Foster City, CA, USA).\(^{14}\) The purity of MPAPO was assayed by HPLC method.

**Stability Assay**

MPAPO, PACAP27, PACAP38, or VIP was respectively dissolved in sodium phosphate buffer (20 mM, pH 8.0) containing 150 mM sodium chloride to obtain a solution at a final concentration of 1 mg/mL and then incubated at 37°C. Samples were analyzed by liquid chromatography mass spectrometry (LC-MS; Applied Biosystems, Foster City, CA, USA) at 1-, 2-, 3-, and 4-week time points (after incubation). The HPLC-ESI-MS system containing a 1.0-×150-mm 300 SB-C18 Sep-Pak column was used to analyze each sample after an injection of 2 μL through increasing concentrations of acetonitrile from 5% to 55% for 35 minutes at 0.05 mL/min.
A New Recombinant PACAP-Derived Peptide, MPAPO

Competition Receptor Binding Assay

The potential of MPAPO to displace \(^{[125]}\)I-PACAP27 by competitively binding to the human PAC1 receptor was examined in PAC1-CHO cell membrane by the method previously described.\(^{23}\) The half-maximal inhibitory concentration (IC50) of MPAPO, PACAP27, PACAP38, and VIP was detected. \([K_{15,R16,L27} VIP(1–7)/GRF(8–27)], a VPAC1-specific agonist, was used as the negative control in the receptor binding assay.\(^{24}\)

Assay of Cyclic Adenosine Monophosphate (cAMP) Accumulation Induced by MPAPO

PAC1-CHO, VPAC1-CHO, and VPAC2-CHO cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) at 37°C and washed with PBS twice before experimentation. Pituitary adenylate cyclase-activating polypeptide 38 or MPAPO was added to 500-μL cell suspension (2 × 10^6 cells/mL) to obtain a solution at a final peptide concentration from 1 × 10^-11 to 1 × 10^-6 M, and the solutions were incubated at 37°C for 5 minutes. One microliter 0.2 M HCl was added and the mixtures were incubated at 37°C for 30 minutes. Cyclic AMP concentrations induced by MPAPO or PACAP27 were measured by the method previously described\(^{23}\) using the cAMP enzyme immunoassay kit (Cayman Chemical Company, Ann Arbor, MI, USA).

Effect of MPAPO on Corneal Wound Repairing and Trauma-Related Protein Expression

Twenty-four mice were randomly divided into four groups (six mice per group), and central corneal wounding was performed as previously described.\(^{25}\) Then mice were treated with 20 μL sterile normal saline (NS, vehicle), 1 nM recombinant bovine (rb)-bFGF, PACAP27, and MPAPO solution, respectively. Mice were treated with NS or different peptides once every 6 hours. Before administration and 12, 24, 36, and 48 hours after the first administration, 1% sterile fluorescein sodium was added to the injured corneal epithelium. After 3 seconds, ocular surfaces were rinsed with sterile saline solution and quickly photographed with a digital camera (Eastman Kodak, Rochester, NY, USA). Assessment of wound closure used fluorescein staining of the ocular surface and digital analysis of the stained area. The images were examined with the software Image-Pro Plus ver. 4.0 (Planetron, Tokyo, Japan). The corneal wound area and the repair ratio [formula: repair ratio = (original wound area − wound area at each time point)/original wound area] were analyzed.

Two hundred Japanese white rabbits were divided into seven groups (six rabbits per group, female and male rabbits each half): NS and 0.2, 0.5, and 1.0 nM MPAPO and PACAP27 groups. The rabbits were subjected to topical anesthesia with 10 μL 0.4% benoxinate hydrochloride in a single eye, and Schirmer tear test was performed using standard sterile strips. One millimeter of the rounded end of the strip was folded and inserted into the lower eyelid; the strip was removed after 5 minutes, and the length of the wet portion below the folded end was immediately measured. Before administration and 10, 30, 60, and 120 minutes after administration of 50 μL NS or various concentrations of MPAPO and PACAP27 solution, the wet strip lengths were determined, and the length differences between pre- and postadministration represented the change in lacrimal secretion.

Another 42 Japanese white rabbits were grouped and treated with the same methods as explained above. The lacrimal fluids were collected into a precooled eppendorf (EP) tube with a pipetter at 30, 30, 60, and 120 minutes after administration of 50 μL NS or various concentrations of MPAPO and PACAP27 solution. The protein concentrations in each experimental group were determined with BCA protein assay kit (Invitrogen). The protein secretion ratios at each time point after administration were calculated.

Forty-two Japanese white rabbits were divided into seven groups (six rabbits per group) and treated with 50 μL NS, rb-bFGF, PACAP27, and MPAPO (1 nM) solution, respectively, after alkali burn to each rabbit’s right central cornea. Anesthesia in the Japanese white rabbits was induced with intramuscular injections of ketamine hydrochloride, 50 mg/kg, and chlorpromazine, 10 mg/kg. Benoxinate hydrochloride (0.4%) was used for topical anesthesia, and surplus moisture was removed with a cotton-tipped applicator. Six-millimeter filter paper discs were soaked for 3 seconds in 0.5 M NaOH solution, then applied to the central cornea for 30 seconds, resulting in the right central cornea alkali burn model. Rabbits were continuously treated for 14 days with NS or different peptides once every 8 hours. Corneal neovascularization (CNV) was examined on day 7 and 14 after administration by slit-lamp biomicroscopy (Oakland, NJ, USA), and the length of the least tortuous and longest new blood vessel that had grown into the center of the corneal opacity was defined as the length of CNV. The area of CNV was calculated using the following formula: \[ C = \frac{4}{3} \pi \frac{r^2 - (r - \frac{L}{2})^2}{L} \], where \( C \) is the circumference of CNV coverage on the cornea, \( r \) is the radius of the selected vessel, and \( L \) is the length of the selected vessel.

Assay of MPAPO Affecting the Proliferation of Mouse Corneal Epithelial Cells

The oxidative injury corneal epithelial cell model was established with H2O2. Normal mouse corneal epithelial cells were seeded on a 96-well culture plate at 3000 cells/well. After 80% of the cells adhered to the wall, cells were treated with various concentration of H2O2 (1, 5, 10, 20, 50, 100, 200, and 500 μM) for 10 minutes, 30 minutes, and 1, 6, and 12 hours, respectively. Treatment without H2O2 was used as control. The
survival of the cells in each experimental group was determined by the tetrazolium-based colorimetric assay (MTT assay; Sigma-Aldrich Corp., St. Louis, MO, USA). The condition under which cell survival was approximately 50% was selected for establishing the H₂O₂-injured corneal epithelial cell model.

The normal or H₂O₂-injured mouse corneal epithelial cells were respectively seeded in a 96-well culture plate at 5000 cells/well. The cells were treated with 0.1, 0.2, 0.5, 1.0, and 5.0 nM PACAP27 or MPAPO for 24 hours, respectively (PBS was used as control). The proliferation rates of the cells in each experimental group were determined using the MTT method. In the PAC1 receptor blocking experiments, cells were preincubated with 100 nM D(24-42)Maxadilan (DMax; Sigma-Aldrich, Shanghai, China), a PAC1 receptor antagonist, for 30 minutes at 37°C before adding 1 nM MPAPO.

**Immunofluorescence Assay for the Effects of MPAPO on Synapse Growth of Mouse Trigeminal Ganglion Cells**

C57BL/6 mice, 1 month of age, were euthanized by neck dislocation. The bilateral trigeminal ganglion cells were taken, washed with DMEM/F12 medium, then digested using enzymolysis (2 g/L collagenase I and 0.1 g/L trypsin III). The cell suspensions were filtrated into a 35-mm petri dish through a 200-mesh nylon screen. The mouse trigeminal ganglion cells were identified with flow cytometry, then seeded onto a pretreated cover glass that contained polylysine in a culture plate at 1 × 10⁵ cells/mL. Cells were treated for 48 hours with media containing 0.1 and 1 nM MPAPO or PACAP27, respectively. The cover glass was fixed and incubated with NF-200 (Abcam, Cambridge, MA, USA) and IgG-FITC antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA); then 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) staining and immunofluorescence detection were carried out.

**Gene Microarrays or Western Blot Assay in Normal and H₂O₂-Injured Trigeminal Ganglion Cells Treated With or Without MPAPO**

The mouse trigeminal ganglion cells cultured with DMEM/F12 media were treated with 100 μM H₂O₂ for 6 hours. When the cell survival reaching approximately 70%, H₂O₂-injured trigeminal ganglion cells were treated with 1 nM MPAPO for 48 hours. Normal and H₂O₂-injured trigeminal ganglion cells treated with or without MPAPO were lysed by adding Trizol reagent, and total RNA of three groups of cells was obtained using Qiagen RNeasy mini kit and Qiagen RNase-free DNase. Gene microarrays or Western Blot Assay in Normal and H₂O₂-Injured Trigeminal Ganglion Cells Treated With or Without MPAPO shows that the binding energy between MPAPO and PAC1 is 91 kCal/mol, which is lower than that of PACAP27 for PAC1, and VIP were remarkably diminished and the slower-migrating peak emerged, probably as a result of peptide degradation. On the other hand, MPAPO exhibited dramatic improvement in stability, losing only 6.7% of the main peak. The stability was increased approximately 31-fold compared with that of PACAP27, PACAP38, and VIP in vitro (Fig. 2A).

**Statistical Analysis**

Results are presented as mean ± SEM of at least three independent experiments. Differences between groups were analyzed using analysis of variance with SPSS version 15.0 (International Business Machines Corporation, Armonk, New York, USA). Post hoc analysis was used if the analysis of variance was significant. A value of P <0.05 was considered statistically significant.

**Results**

**Preparation and Bioinformatics Analysis for Recombinant MPAPO**

The DNA fragment encoding 29 amino acids of MPAPO was directly ligated to a gel-purified NdeI/SapI-digested pKYB-MCS vector (New England Biolabs, Ipswich, MA, USA) to yield the recombinant expression plasmid pKY-MPAPO (Figs. 1A, 1B). The fusion proteins comprising MPAPO, intein, and chitin-binding domain (i.e., MPAPO-intein-CBD) were expressed through the recombinant vector pKY-MPAPO in E. coli strain ER2566 and purified using chitin affinity column (New England Biolabs). The cleavage of intein was induced by β-mercaptoethanol and the target peptide, MPAPO, was released. Then the recombinant MPAPO was further purified and prepared by reverse-phase HPLC. Approximately 29.6 mg recombinant MPAPO peptide can be obtained from 1 L Luria-Bertani medium. The prepared MPAPO was analyzed and identified by tricine-SDS-PAGE (Fig. 1C) and ESI-MS (Fig. 1D). The molecular weight of MPAPO obtained by ESI-MS was 3405.0 Da, which was consistent with the theoretical value. The purity of prepared MPAPO was over 96% by the analytical HPLC determination method (Fig. 1E).

PAC1 receptor belongs to class II 7-transmembrane domain G protein-coupled receptor family (GPCRs). The structure of PAC1 receptor comprises extracellular, transmembrane, and intracellular domain, and PACAP binds to the extracellular domain. The three-dimensional structure of PAC1 receptor was modeled through homology modeling (Fig. 1F). Binding studies between PAC1 receptor and MPAPO or PACAP27 were conducted by using protein–protein docking software including ZDOCK and RDOCK protocol (Fig. 1G). Data analysis shows that the binding energy between MPAPO and PAC1 is ~91 kCal/mol, which is lower than that of PACAP27 for PAC1, ~80 kCal/mol. Compared with PACAP27, MPAPO may have higher affinity and selectivity for PAC1 receptor.

**MPAPO Has Higher Stability and Selectively Activates PAC1 Receptor With Higher Potency and Bioactivity Than Wild PACAP27**

The stability of PACAP, VIP, and the recombinant MPAPO was measured at 37°C in sodium phosphate buffer (20 mM, pH 8.0) containing 150 mM sodium chloride. After incubation for 4 weeks at 37°C, the main peptide peaks for PACAP27, PACAP38, and VIP were remarkably diminished and the slower-migrating peak emerged, probably as a result of peptide degradation. On the other hand, MPAPO exhibited dramatic improvement in stability, losing only 6.7% of the main peak. The stability was increased approximately 31-fold compared with that of PACAP27, PACAP38, and VIP in vitro (Fig. 2A).
FIGURE 1. Preparation and identification of MPAPO and bioinformatics analysis for the binding with PAC1 receptor. (A) The amino acid sequence of MPAPO and PACAP27. (B) Map of the recombinant expression vector pKY-MPAPO. SDS-PAGE (C) and ESI-MS (D) of the prepared recombinant MPAPO. (E) HPLC of the prepared recombinant MPAPO. Prepared MPAPO at 1 mg/mL in 45% acetonitrile containing 0.1% trifluoroacetic acid (TFA) was analyzed by analytical HPLC. (F) Homology modeling of full-length human PAC1 receptor (hPAC1) using the software package Discovery Studio 2.5.5. (G) Binding affinity analysis of MPAPO to full-length hPAC1 by protein–protein docking. SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ESI-MS, electrospray ionization mass spectrometry; HPLC, high-performance liquid chromatography.
Human PAC1 receptor-transfected CHO cells (PAC1-CHO) were used for competition receptor binding assay. Competition binding of [125I]PACAP27 on membranes purified from CHO cells identified MPAPO as a PAC1-specific peptide. MPAPO competitively displaced [125I]PACAP27 from PAC1 with a half-maximal inhibitory concentration (IC50) of 9.0 ± 7.2 nM, and the IC50 of the wild PACAP27 and VIP was 18.0 ± 4.8 nM and 19.1 ± 2.2 μM, respectively (Fig. 2B). In contrast, the IC50 for VIP(1–7)/GRF(8–27), an established human VPAC1-specific agonist, for human PAC1 was more than 20 nM. These results showed that MPAPO could competitively displace [125I]PACAP27 by binding to human PAC1 receptor in PAC1-CHO cells and the IC50 of MPAPO was significantly lower than that of wild PACAP27 and VIP.

The accumulation of cAMP in human PACAP receptor-transfected cells (PAC1-CHO, VPAC1-CHO, and VPAC2-CHO) was used as an index of the agonist activity. MPAPO was a potent agonist for the PAC1 with a half-maximal stimulatory concentration (EC50) of 0.21 nM. However, the receptor potency of MPAPO for human VPAC1 (EC50 of 28.9 nM) and VPAC2 (EC50 of 33.7 nM) was only 1/138 and 1/161 of that for human PAC1, respectively. Compared with MPAPO, the EC50 for PACAP27 for human PAC1, VPAC1, and VPAC2 receptor was 0.59, 1.08, and 1.19 nM, respectively (Fig. 2C). These results showed that MPAPO was a PAC1-specific agonist with high potency and bioactivity (Fig. 2D), and receptor potency of MPAPO was significantly higher than that of wild PACAP27.

**MPAPO Can Quickly Perform Corneal Epithelial Wound Closure and Affect Trauma-Related Protein Expression in C57BL/6 Mice**

After the C57BL/6 mice with central corneal wound were treated with sterile NS (vehicle), rb-bFGF, PACAP27, and MPAPO, corneal epithelial cells were stained with fluorescein sodium at different time points. The results showed that the injured area stained yellow-green was continuously and significantly decreased over time in MPAPO-treated mice. At 12 hours after the first administration, the repair rate of the injured corneas reached 49.58% in MPAPO-treated mice; however, the repair rate was only 9.76%, 36.75%, and 38.79% in NS-, rb-bFGF-, and PACAP27-treated mice, respectively. MPAPO carried out a complete wound closure but not NS, rb-bFGF, and PACAP27 at 30 hours after the first administration (Figs. 3A, 3B).

Expression of trauma-related protein such as NGF, FN, TGF-β, ICAM-1, and VEGF was determined by ELISA at different time points after the first administration. The results showed that, compared with normal mice and NS-treated mice, the expression level of NGF, TGF-β, and FN, three proteins conducive to wound repair, was significantly higher in MPAPO- and PACAP27-treated mice, respectively. MPAPO significantly promoted cell proliferation from 0.1 to 1 nM in a dose-dependent manner, and the cell proliferation rate reached 70%, which was obviously higher than that for NS and PACAP27 (Fig. 5A).

**MPAPO Can Improve Lacrimal Gland Secretion and Inhibit Corneal Neovascularization in Japanese White Rabbits**

The Japanese white rabbits were treated with NS and 0.2, 0.5, and 1 nM MPAPO or PACAP27 for 10, 30, 60, and 120 minutes; then lacrimal fluids were collected within 5 minutes after administration, and the lacrimal fluid secretion amounts and lacrimal protein secretion rate were determined. The results showed that MPAPO could significantly increase the lacrimal fluid secretion amount in a dose-dependent manner (Fig. 4A). In rabbits receiving 1 nM MPAPO for 60 minutes, the lacrimal fluid secretion amount was largest and was 222.9% and 57.8% higher than that for NS and PACAP27 (Fig. 4A), respectively. Furthermore, the total protein concentration in the lacrimal fluids was also significantly increased over time in MPAPO-treated rabbits. The protein-increasing effects of MPAPO were observed as early as 10 minutes after administration, and the lacrimal protein secretion rate reaching maximum in 0.5 nM MPAPO-treated rabbits at 30 minutes after administration was, respectively, 81.7% and 52.6% higher than that in NS- and PACAP27-treated rabbits (Fig. 4B). These results showed that MPAPO can significantly improve lacrimal gland secretion, and the increase of lacrimal protein may efficiently ameliorate dry eye induced by injury.

The CNV in NS-, MPAPO-, PACAP27-, and rb-bFGF-treated eyes with central corneal alkali burn was examined on day 7 and 14 after administration. The differences in CNV between MPAPO- and NS-treated rabbits were evident on days 7 and 14, with most of the control eyes showing CNV invading the central cornea and not the MPAPO-treated eyes. The eye number with CNV in MPAPO-treated eyes was 6% (3 of 50) on day 7 and 10% (5 of 50) on day 14, whereas it was 18% and 48% for NS, 12% and 38% for rb-bFGF, and 10% and 50% for PACAP27 on day 7 and 14, respectively (Table 1). Areas of CNV in MPAPO-treated eyes were 11.5 ± 1.8 mm² on day 7 and 12.2 ± 1.9 mm² on day 14, values that were significantly lower than those in NS-, PACAP27-, and rb-bFGF-treated eyes, and CNV inhibition of MPAPO reached 66.8% on day 7 and 71.2% on day 14, values that were obviously higher than for rb-bFGF and PACAP27 (Table 1). These results showed that MPAPO can significantly inhibit CNV induced by alkali burn, and the effect was stronger than for rb-bFGF and PACAP27.

**MPAPO Significantly Promotes Mouse Corneal Epithelial Cell Proliferation**

The normal mouse corneal epithelial cells were treated with PBS and 0.1, 0.2, 0.5, 1, and 5 nM MPAPO or PACAP27 for 24 hours (PBS was used for control), and the cell proliferation rates were measured by MTT method. The results demonstrated that MPAPO can promote normal mouse corneal epithelial cell proliferation below 1 nM in a dose-dependent manner, and the proliferation rate of cells treated with 1 nM MPAPO was up to 70%, which was obviously higher than that for PACAP27 (Fig. 5A).

The normal mouse corneal epithelial cells were treated with various concentrations of hydrogen peroxide (H₂O₂) for 10 minutes, 30 minutes, and 1, 6, and 12 hours, and the cell survival rates under different injury conditions were calculated. The results showed that the survival rate was approximately 50% when the cells were treated with 200 μM H₂O₂ for 10 minutes (Fig. 5B). The H₂O₂-injured corneal epithelial cell model was established with this condition and was treated with PBS or 0.1, 0.2, 0.5, 1, and 5 nM MPAPO or PACAP27 for 24 hours. The MTT assay results showed that MPAPO can significantly promote cell proliferation from 0.1 to 1 nM in a dose-dependent manner, and the cell proliferation rate reached...
approximately 40% for 1 nM MPAPO-treated cells (Fig. 5C). In contrast, the cell proliferation-promoting effects disappeared in normal or H₂O₂-injured mouse corneal epithelial cells preincubated with 100 nM DMax, a PAC1 receptor antagonist, before 1 nM MPAPO or PACAP27 treatment (Figs. 5A, 5C).

MPAPO Can Significantly Increase Synapse Formation of Trigeminal Ganglion Cells

The trigeminal ganglion cells were separated from C57BL/6 mice and cultured. Then the cells were purified with serum-free medium. The trigeminal ganglion cells were harvested on day 13 and identified using neuron-specific enolase (NSE) as a label with flow cytometry; the amount of NSE-positive cells reached 98.40% (Fig. 5D). The mouse trigeminal ganglion cells were treated with 0.1 and 1 nM MPAPO or PACAP27 for 48 hours, and the NF-200 immunofluorescence assay results showed that MPAPO can significantly promote synapse growth in mouse trigeminal ganglion cells in a dose–effect manner (Fig. 6A). The immunofluorescence quantitative analysis for NF-200 showed that the fluorescence intensity in cells treated with 0.1 and 1 nM MPAPO was 1.56- and 2.09-fold that for PACAP27, respectively (Fig. 6B), whereas the fluorescence intensity was significantly reduced in DMax-treated cells before 1 nM MPAPO treatment. The results demonstrated that MPAPO can significantly promote synapse growth in mouse trigeminal ganglion cells through PAC1 activation, and the biological effects were significantly higher than those for PACAP27.

MPAPO-Promoted Corneal Nerve Injury Repair May Relate to MAPK Signaling Pathway

Mouse normal and H₂O₂-injured trigeminal ganglion cells were respectively treated with PBS or 1 nM MPAPO for 48 hours, and the cell gene expression was analyzed with GeneChip (Affymetrix, Santa Clara, CA, USA). The results showed that after MPAPO administration, some gene expressions in several signaling pathways had significant difference. The number of differential expression genes reached 16 in mitogen-activated protein kinases (MAPK) signaling pathway (Table 2), a key signal transduction pathway associated with trauma. Furthermore, Western blot assay results demonstrated that MPAPO could significantly increase expression of Akt, p-Akt (Thr308), Erk1/2, and p-Erk1/2 in normal trigeminal ganglion cells (Fig. 7A). In the H₂O₂-injured trigeminal ganglion cells, even without MPAPO treatment, expression levels of p-Erk1/2 and p-Akt (Thr308) were higher than in normal cells, which may relate to the emergency reaction after injury. The expression of p-Akt (Thr308) and p-Erk1/2 was 1.28-fold and 0.75-fold higher in MPAPO-treated cells than PBS-treated controls, whereas there was only a 0.83- and 0.44-fold increase in the expression of p-Akt (Thr308) and p-Erk1/2 in PACAP27-treated cells, respectively—values significantly smaller than in MPAPO-treated cells (Fig. 7B). These expression or phosphorylation level increases could further activate the downstream signal transduction, which could effectively promote synapse growth and proliferation of neural cells, thus significantly facilitating the functional recovery of corneal and trigeminal nerve after injury.

**DISCUSSION**

The cornea is a vital component of the eye because it provides approximately 70% of the refraction and focusing of incoming light. Unlike most tissues in vivo, the cornea contains no blood vessels to nourish or protect it against infection. Instead, the cornea receives its nourishment from tears and aqueous humor, furthermore supplied by the terminal branches of the
FIGURE 3. Effect of recombinant MPAPO on injured corneal epithelial repairing and the expression of the trauma-related protein in C57BL/6 mice.

(A) MPAPO efficiently promotes central corneal epithelial wound closure. Change in wound area stained by sodium fluorescein within 48 hours in different treated groups.

(B) The rates of injured corneal repair in sterile normal saline (NS, vehicle), rb-bFGF, PACAP27-, or MPAPO-treated mice.

(C) Effect of recombinant MPAPO on the expression of NGF, TGF-β, FN, ICAM-1, and VEGF in mouse model with central corneal wound. *P < 0.05, **P < 0.01, rb-bFGF, PACAP27, or MPAPO versus NS; #P < 0.05, MPAPO versus PACAP27; ##P < 0.01, MPAPO versus rb-bFGF, MPAPO versus PACAP27 (Scheffé test, n = 5).
ophthalmic division of the trigeminal nerve. It was found that exogenous protein was taken up in axonal branches in the ophthalmic nerve and transported back to the trigeminal ganglion as has been suggested for certain viral infections. When corneal injury occurs, many terminal branches of the sensory nerves are cut or damaged; thus the cornea’s main function and transparency are severely damaged. Corneal injury can also induce dry eye, and in turn dry eye may aggravate postinjury neovascularization and inflammation.

Several therapeutics against corneal injury have been explored; and some cytokines, such as basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), keratinocyte growth factor, vascular endothelial factor (VEGF), and pigment epithelium-derived factor (PEDF), have been found to stimulate corneal epithelial cell migration and wound closure in vivo. However, with bFGF or EGF acting as the main therapeutics, bFGF- or EGF-induced corneal angiogenesis, which is currently untreatable, is a significant side effect.

**FIGURE 4.** MPAPO efficiently increases lacrimal fluid and lacrimal protein secretion in Japanese white rabbits. (A) Lacrimal fluid secretion of Japanese white rabbits respectively treated with NS or 0.2, 0.5, and 1 nM MPAPO or PACAP27 for 10, 30, 60, and 120 minutes, and comparison of lacrimal fluid secretion caused by MPAPO or PACAP27 at 60 minutes after administration. (B) Lacrimal protein secretion rate of Japanese white rabbits treated with NS or 0.2, 0.5, or 1 nM MPAPO or PACAP27 for 10, 30, 60, and 120 minutes, and comparison of lacrimal protein secretion rate by MPAPO or PACAP27 at 60 minutes after administration. *P < 0.05, **P < 0.01, MPAPO, PACAP27, or rb-bFGF versus NS; #P < 0.05, ##P < 0.01, MPAPO versus PACAP27, MPAPO versus rb-bFGF (Scheffé test, n = 6).
Thus for repair of corneal injury, effectively promoting wound closure, adequate corneal innervation, and neovascularization inhibition is key to restoring normal function.

PAC1, as the specific receptor for PACAP, is widely distributed on the membranes of target cells such as corneal epithelial cells and trigeminal ganglion cells; PACAP thereby plays biological roles such as promoting cell proliferation, growth, development, differentiation, and injury repair, mainly via PAC1 receptor activating the cAMP-dependent protein kinase (PKA) signal pathway and phosphatidylinositol signal transduction pathway. In addition, PACAP has been reported to have potential anti-inflammatory activity that could be beneficial in the setting of corneal injury. However, the first two N-terminal amino acids of wild PACAP and some of its derivates are H-S-, which is sensitive recognition and cleavage site of the DPP4, and it has been confirmed that DPP4 is widely distributed in ocular tissues, especially corneal epithelium and endothelium. The PACAP segment that lost the N-terminal sequence H-S- converted PACAP from an agonist of PAC1 to an antagonist with low binding affinity. Furthermore, the peptides were poorly stable in aqueous solution and easily degradable; thereby their therapeutic application has been hampered by the short half-life and limited bioavailability in vivo.

In the current work, compared with wild PACAP27, the new recombinant MPAPO with four mutations was generated using gene recombination technique. A methionine was additionally added to the N-terminus of MPAPO in order to block the N-terminus and prevent loss of the sequence H-S- susceptible to enzyme digestion by DPP4. The M17L mutation was introduced in an effort to eliminate oxidation, which was present in wild PACAP, contributing to destabilization in vivo. Bioinformatics analysis showed that the L27K mutation and addition of a lysine to the C-terminus would contribute to the higher affinity or specificity of MPAPO for PAC1 compared to wild PACAP (data not shown). Consistent with this, the experimental results showed that MPAPO was a PAC1-specific agonist with higher stability and receptor potency than wild PACAP. In the mouse model with corneal injury, MPAPO can quickly perform corneal

<table>
<thead>
<tr>
<th>Eye Number With CNV, %</th>
<th>Area of CNV, mm²</th>
<th>CNV Inhibition, %</th>
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<tbody>
<tr>
<td>Day After Treatment</td>
<td>NS MPAPO PACAP27 rb-bFGF</td>
<td>NS MPAPO PACAP27 rb-bFGF</td>
</tr>
<tr>
<td>Seventh day</td>
<td>18 6†‡ 10† 12* 34.7 ± 6.2 11.5 ± 1.8†‡ 20.8 ± 3.2† 29.2 ± 2.7*</td>
<td>66.8† 40.1 15.9</td>
</tr>
<tr>
<td>14th day</td>
<td>48 10†‡ 30† 38* 42.5 ± 8.1 12.2 ± 1.9†‡ 26.2 ± 4.1† 36.7 ± 4.9*</td>
<td>71.2† 38.4 13.7</td>
</tr>
</tbody>
</table>

* P < 0.05, rb-bFGF versus sterile normal saline (NS, vehicle).
† P < 0.01, PACAP27 or MPAPO versus NS.
‡ P < 0.01, MPAPO versus rb-bFGF, MPAPO versus PACAP27.

**TABLE 1. Comparison of Corneal Neovascularization (CNV) in NS-, MPAPO-, PACAP27-, and rb-bFGF-Treated Eyes With Central Corneal Alkali Burn on Days 7 and 14 After Administration**

**FIGURE 5.** Effects of recombinant MPAPO on normal or H₂O₂-injured murine corneal epithelial cell proliferation and the identification of the separated trigeminal ganglion cells from C57BL/6 mice. (A) Proliferation rates of normal murine corneal epithelial cells treated by PBS or 0.1, 0.2, 0.5, 1, and 5 nM MPAPO and PACAP27, respectively. (B) Effects of H₂O₂ on murine corneal epithelial cell viability. Cell survival rate (%) = average optical density at 450 nm (OD₄₅₀) of each experimental group/average OD₄₅₀ of PBS group. (C) Proliferation rates of H₂O₂-injured murine corneal epithelial cell model treated by PBS or 0.1, 0.2, 0.5, 1, and 5 nM MPAPO and PACAP27. (D) The harvested trigeminal ganglion cells were identified by neuron-specific enolase (NSE), a specific marker, and flow cytometry. PAC1 receptor antagonist D(24-42)Maxadilan (DMax) was used for blocking PAC1 receptor, and the cells were preincubated with 100 nM DMax for 30 minutes at 37 °C before 1 nM MPAPO treatment in (A) and (C). #P < 0.05, ##P < 0.01, MPAPO versus PACAP27 (Scheffe test, n = 3).
FIGURE 6. MPAPO efficiently promotes synapse formation in the trigeminal ganglion cells of C57BL/6 mice. (A) NF-200 immunofluorescence was detected in mouse trigeminal ganglion cells treated with PBS or 0.1 and 1 nM MPAPO or PACAP27. (B) NF-200 immunofluorescence quantitative analysis with Image-Pro Plus system. **P < 0.01, MPAPO or PACAP27 versus PBS; ###P < 0.01, MPAPO versus PACAP27 (Scheffé test, n = 3).
epithelial wound closure, and the repair effect was stronger than that for wild PACAP27 and rb-bFGF, an anti-corneal wound clinical drug. MPAPO can also significantly increase the expression of NGF, TGF-β, and FN while inhibiting high expression of ICAM-1 and VEGF after corneal injury. Nerve growth factor can promote corneal epithelial cell proliferation and migration and accelerate corneal wound healing by stimulating the release of various neuroepitides or growth factors; in addition, NGF plays an important role in sensory neuron survival as a neurotrophic factor. Fibronectin is not only the marker protein for reactive wound repairing/healing, but also an extracellular protein for enhancing nerve repair. Transforming growth factor-β can increase the expression of extracellular matrix genes, such as FN, and various collagens and proteinase inhibitors. However, there was little or no ICAM-1 expression in the normal cornea, abnormally high expression occurred after corneal injury, thereby inducing the inflammatory and VEGF-dependent CNV. Corneal neovascularization can be significantly decreased by specifically inhibiting VEGF (Western blot test); this can efficiently promote the survival and differentiation of the neurons, as well as nerve injury repair through the PI3K-Akt, Erk, or p38MAPK signal transduction pathways.

The use of proteins and peptides as human therapeutics has increased rapidly in recent years. Peptides are considered viable therapeutic agents because of their superior specificity compared to small molecules and their smaller size relative to antibodies. In addition, we have optimized the expression and preparation conditions for MPAPO so that the MPAPO yield can reach 30 mg/L (Luria-Bertani culture), and purity can reach more than 96%. As compared with the commonly used chemically synthesized PACAP and derivates, the cost can be reduced by approximately 60%.

In summary, our study provides the new recombinant PACAP-derived peptide MPAPO, with higher stability, which exhibits potent effects in promoting corneal wound repair and lacrimal secretion without neovascularization via selective activation of PAC1 receptor. Currently, clinical therapeutics with few side effects for corneal injury are rare, so the recombinant MPAPO has potential to be developed into an external therapeutic against corneal injury or dry eye.

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References


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