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Research article

Diquafosol promotes corneal epithelial healing via intracellular calcium-mediated ERK activation

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ABSTRACT

Diquafosol is known as a purinergic P2Y2 receptor (P2Y2R) agonist that stimulates water and mucin secretion from conjunctival epithelial cells and goblet cells, leading to tear film stability in dry eye. However, its effect on corneal epithelial healing has not yet been elucidated. The aim of the present study was to evaluate the effect of diquafosol on corneal epithelial healing *in vivo* and on P2Y2R-related downstream signaling pathways *in vitro*. We administered 3% diquafosol ophthalmic solution on 3 mm-diameter epithelial defects made in rat corneas and assessed the wound closure over time. Corneal epithelial healing was significantly accelerated in diquafosol-treated eyes compared to control eyes at 12 and 24 h. During wound healing, P2Y2R staining appeared stronger in the re-epithelized margin near the wound defect. To evaluate whether diquafosol stimulates epidermal growth factor receptor/extracellular-signal-regulated kinase (EGFR/ERK)-related cell proliferation and migration, simian virus 40-transfected human corneal epithelial (THCE) cells were used for *in vitro* experiments. Cell proliferation was accelerated by diquafosol at concentrations from 20 to 200 μM during 48 h, but inhibited at concentrations over 2000 μM. The intracellular calcium ($[Ca^{2+}]i$) elevation was measured in diquafosol (100 μM)-stimulated cells using Fluo-4/AM ($[Ca^{2+}]i$ indicator). $[Ca^{2+}]i$ elevation was observed in diquafosol-stimulated cells regardless of the presence of calcium in media, and suramin pretreatment inhibited the calcium response. The effect of diquafosol on phosphorylation of EGFR, ERK and Akt, and cell migration was determined by western blotting and *in vitro* cell migration assay. Diquafosol induced phosphorylation of EGFR at 2 min post-stimulation, and phosphorylation of ERK at 5 min post-stimulation. Phosphorylation of ERK was attenuated in cells pretreated with suramin or BAPTA/AM ($[Ca^{2+}]i$ chelator), and partially with AG1478 (EGFR inhibitor). Likewise, diquafosol-treated cells showed acceleration of gap closure in cell migration assay, which was inhibited by suramin, BAPTA/AM, AG1478, and U0126 (MEK inhibitor). These studies demonstrate that diquafosol is effective in promoting corneal epithelial wound healing and that this effect may result from ERK-stimulated cell proliferation and migration via P2Y2R-mediated $[Ca^{2+}]i$ elevation.

1. Introduction

The epithelial layer is the outermost layer to protect the tissue against external stimuli such as mechanical or chemical injury or infection by pathogens (Sack et al., 2001; Thoft et al., 1979). In the cornea, the epithelium is crucial for maintenance of transparency and clear vision by forming a smooth refractive surface covered with tear film (Argueso and Gipson, 2001; Pflugfelder et al., 2000). Corneal epithelial defects must be rapidly restored to avoid pathogen invasion and further damage to the inner layer, which can lead to corneal opacity and vision loss. Significant efforts have been made to understand the mechanisms of epithelial healing and to identify the roles of various growth factors, including epidermal growth factor (EGF), hepatocyte growth factor (HGF), and platelet derived growth factor (PDGF) (Imanishi et al., 2000; Lu et al., 2001; Yu et al., 2010).

Alongside these growth factors, extracellular nucleotides like

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ATP and UTP play roles in epithelial wound healing (Boucher et al., 2007; Crooke et al., 2008, 2009; Dixon et al., 1999; Guzman-Aranguez et al., 2013; Klepeis et al., 2004; Pintor et al., 2004; Weinger et al., 2005). Although nucleotides, particularly ATP, function as a universal energy source, they play a completely different role in the extracellular compartment through the activation of receptors on the cell surface, P2 purinergic receptors (P2Rs). P2Rs are divided into two families based on structural and physiological differences: ionotropic P2X receptors (P2XRs), which are nucleotide-gated ion channels, and metabotropic P2Y receptors (P2YRs), which are G protein-coupled receptors (Junger, 2011). Extracellular nucleotides activate P2YRs and induce downstream signaling events including the mobilization of intracellular calcium ($[Ca^{2+}]i$) and the phosphorylation of EGF receptor (EGFR) and ERK, which elicits the epithelial wound healing response (Boucher et al., 2010; Gendaszewska-Darmach and Kucharska, 2011; Klepeis et al., 2004; Pintor et al., 2004; Yin et al., 2007).

Diquafosol tetrasodium, a stabilized derivative of a UTP dimer (P1,P4-bis[5′-uridyl] tetraphosphate), was recently introduced for treating dry eye. Diquafosol exhibits agonist activity on P2Y2 receptor (P2Y2R), a target receptor of UTP (Pendergast et al., 2001). At the ocular surface, P2Y2R is expressed in the cornea, conjunctiva including goblet cells, and meibomian gland (Cowlen et al., 2003). In the conjunctival epithelium, diquafosol accelerates fluid efflux via chloride channel activation after increasing $[Ca^{2+}]i$. In addition, diquafosol reportedly facilitates the secretion of soluble mucin from conjunctival goblet cells (Fujihara et al., 2002). These actions of diquafosol ophthalmic solution promote tear film stability on the ocular surface, and clinical improvements in the treatment of dry eye using diquafosol ophthalmic solution have been reported in several studies (Matsumoto et al., 2012; Takamura et al., 2012).

However, no previous studies have evaluated the effects of diquafosol on corneal epithelial wound healing. Activation of P2Y2R triggers cell migration in different cell types (Bagchi et al., 2005; Chaulet et al., 2001; Kaczmarek et al., 2005; Pillois et al., 2002). In particular, extracellular nucleotides stimulate cell migration in wounded corneal epithelium via P2Y2R activation (Boucher et al., 2010; Yin et al., 2007). We hypothesized that diquafosol, a UTP dimer, promotes corneal epithelial wound healing through P2Y2R activation and downstream signaling events. In the present study, we demonstrated for the first time that diquafosol promotes corneal epithelial wound healing *in vivo* through animal experiments and that this effect is mediated by downstream signaling events including $[Ca^{2+}]i$ elevation and phosphorylation of EGFR and ERK.

2. Materials and methods

2.1. In vivo animal experiments

Male Sprague–Dawley rats (10–12 weeks old, weighing approximately 300 g) were used in the present study. All surgical procedures were performed in the semi-pathogen free zone of our animal care facility. All experiments were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and approved by the Institutional Animal Care and Use Committee (IACUC) at the College of Medicine, The Catholic University of Korea.

2.1.1. Corneal epithelial wound healing and topical application of diquafosol on the cornea

We evaluated the effect of topical diquafosol on *in vivo* wound healing of the rat corneal epithelium. Prior to surgery, rats were anesthetized with 50 mg/kg tiletamine plus zolazepam (Zoletil®, Virbac, Carros, France) and 15 mg/kg xylazine hydrochloride (Rompun®, Bayer, Leverkeusen, Germany) by intraperitoneal injection, and 0.5% proparacaine ophthalmic solution drops (Alcaine®, Alcon, Fort Worth, TX, USA) were administered to the eyes. A round epithelial debridement (3 mm in diameter) was produced at the center of the cornea according to Saika's method with modification (Saika et al., 2000). Briefly, the central corneal epithelium was demarcated with a trephine (3 mm in diameter; Storz, St. Luis, MO, USA) and subsequently removed using a burr (Algerbrush II, Precision Instruments Inc., Farmington Hills, MI, USA) under an operating microscope (OPMI 1/S100, Carl Zeiss Meditec AG, Jena, Germany). After injury to the epithelium, the eye was treated topically four times a day with 3% diquafosol ophthalmic solution (DIQUAS®, Santen Pharmaceutical Co., Ltd., Osaka, Japan) in treated rats ($n = 6$) or with vehicle (balanced salt solution [BSS], Alcon, Fort Worth, TX, USA) in control rats ($n = 6$) under inhalation anesthesia with isoflurane (Forane, JW Pharmaceutical Co., Seoul, Korea). Surgery and treatment were performed in one eye of treated and control rats. Prophylactic antibiotic eye drops (levofloxacin 0.5%; Cravit®, Santen Pharmaceutical Co.) were also applied to the eye four times a day to prevent infection. Images of the epithelial defects stained with lissamine green dye (Green GloTM, HUB Pharmaceuticals, LLC, Rancho Cucamonga, CA, USA) were taken at 0, 12, 24, and 36 h using a digital camera equipped with an operating microscope (OPMI 1/S100, Carl Zeiss Meditec). The area of the epithelial defect was measured from images using ImageJ software (version 1.48, NIH, Bethesda, Maryland, USA). The percentage of wound closure (mean ± standard error of the mean [SEM]) was calculated at each time point using the following equation: wound closure (%) = [(initial defect area − current defect area)/initial defect area].

2.1.2. P2Y2R immunofluorescence staining of the corneal epithelium

Immunofluorescence staining of rat corneas was performed to examine the expression patterns of P2Y2R in wounded and unwounded epithelium. At 12 h after wound infliction, wounded corneas and contralateral unwounded corneas were dissected from treated rats ($n = 3$) and control rats ($n = 3$), and then fixed in 4% paraformaldehyde (PFA) overnight at 4 °C. After washing with fresh 1× PBS, corneas were soaked in 30% sucrose for 24 h, and then embedded in optimum cutting temperature (OCT) compound (Tissue-Tek®, Sakura Fine Technical Co., Ltd., Tokyo, Japan) and stored at −80 °C. Eight-micrometer cryostat sections were placed on silane-coated slides (5116-20F, Muto Pure Chemical, Tokyo, Japan) and kept for 30 min at room temperature (RT) to air dry. Samples on slides were rinsed in PBS and permeabilized in PBS with 0.1% Triton-X (PBST) containing 10% donkey serum and 1% bovine serum albumin (BSA; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 h at RT. The slides were incubated with primary antibodies in dilution buffer (0.1% PBST with 1% BSA) overnight at 4 °C, and subsequently incubated with secondary antibodies in dilution buffer for 2 h at RT. The slides were rinsed three times with PBS after each step. The slides were mounted in mounting medium with 4′,6-diamidino-2-phenylindole (DAPI; Vectashield®, Vector Laboratories, Inc., Burlingame, CA, USA) and examined with a confocal laser scanning microscope (LSM710, Carl Zeiss Meditec). Goat anti-P2Y2R (1:200; sc-15209, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was used as the primary antibody. Secondary antibodies (1:400; Alexa Fluor® 488, Life Technologies Inc., Carlsbad, CA, USA) were matched to the host species of the primary antibodies.

2.2. In vitro experiments

2.2.1. Cell culture

To evaluate the cellular response to diquafosol *in vitro*, we used

simian virus 40-transfected human corneal epithelial (THCE) cells, kindly provided by Dr. Kaoru Araki-Sasaki, Osaka University, Osaka, Japan (Araki-Sasaki et al., 1995). Cells were cultured in keratinocyte serum-free medium (KSFM; 10724-011, Gibco®, Life Technologies Inc.) containing supplements (50 μg/mL bovine pituitary extract, 5.0 ng/mL recombinant human epidermal growth factor; 37000-015, Gibco®, Life Technologies Inc.) and 5 μg/mL gentamicin (15750-078, Life Technologies Inc.) at 37 °C in a humidified atmosphere at 5% CO_2. The medium was changed every 3 days, and cells were subcultured at 80–90% confluence. Prior to each treatment, cells were starved in KSFM without supplements for at least 12 h.

2.2.2. Cell proliferation/cytotoxicity assay

To determine the concentration of diquafosol to be used for the *in vitro* experiments, cell proliferation/cytotoxicity assays were performed using cell proliferation/cytotoxicity kits (CCK-8 kit and WST-8 kit, Dojindo Molecular Technologies, Kumamoto, Japan) according to the manufacturer's protocol. Suspended THCE cells were seeded onto a 96-well plate (1×10^4 cells in 100 μL supplement-free KSFM per well) and incubated with various concentrations of diquafosol (1.0–4.0 mM) for 48 h in a humidified incubator (37 °C, 5% CO_2). KSFM only and KSFM containing recombinant human (rh)-EGF were used as positive and negative controls, respectively. CCK-8 reagent was added to each well of the plate after 30 min, 1, 2, 4, 8, 12, 24, or 48 h. After incubating for an additional 2 h, we measured the absorbance values at 450 nm using a microplate reader (VersaMax™, Molecular Devices, LLC, Sunnyvale, CA, USA). All experiments were conducted in triplicate.

2.2.3. Measurement of $[Ca^{2+}]i$

THCE cell monolayers were grown in a 35-mm dish (Corning® CellBIND®, Corning Inc., NY, USA) and incubated with KSFM containing 4 μM Fluo-4/AM (intracellular calcium indicator; Molecular Probes™, Life Technologies) for 30 min at 37 °C in a humidified incubator. After washing with Hank's balanced salt solution (HBSS; #14170-112, Gibco®, Life Technologies Inc.), the cells were incubated for an additional 30 min in four different media conditions (1 mL): 1) KSFM only (containing 0.1 mM calcium), 2) KSFM with suramin (200 μM), 3) calcium-free KSFM (#10725-018, Gibco®, Life Technologies Inc.) only, and 4) calcium-free KSFM with suramin. Then, diquafosol (200 μM) in loading medium (1 mL calcium-free KSFM) was applied to the dishes. While diquafosol was loaded, fluorescence images (516 nm) were obtained sequentially at 1.0-s intervals for 5 min using a live cell imaging inverted fluorescence microscope (Leica AF6000 Modular System, Leica Microsystems, Wetzlar, Germany). Fluorescence intensity was expressed as relative fluorescent units (F/F0) to initial fluorescent intensity (F0) using ImageJ software (version 1.48, NIH).

2.2.4. Western blot analysis

P2Y2R and phosphorylation of EGFR, ERK1/2, and Akt were quantified by western blot analysis. THCE cells at 80–90% confluence were starved in KSFM without supplements for 12 h, after which they were incubated in KSFM or KSFM containing diquafosol 100 μM, suramin 200 μM (P2Y2 antagonist; Sigma–Aldrich, St. Louis, MO, USA), AG1478 5 μM (EGFR inhibitor; Sigma–Aldrich), BAPTA/AM 5 μM (intracellular calcium chelator, Santa Cruz Biotechnology, Inc.), or U0126 10 μM (MEK inhibitor; Sigma–Aldrich) to inhibit the respective signaling pathways. After each treatment, THCE cells were lysed in RIPA buffer (20 mM Tris–HCl, pH 7.5, 0.1% [w/v] sodium lauryl sulfate, 0.5% [w/v] sodium deoxycholate, 135 mM NaCl, 1% [v/v] Triton X-100, 10% [v/v] glycerol, and 2 mM EDTA) supplemented with a protease and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL, USA). Cell lysates were centrifuged at 14,000 rpm for 15 min and the supernatants were collected. Total protein concentrations were determined via BCA protein assay. The lysates were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and then blotted onto a 0.45-μm nitrocellulose membrane (Amersham™, Protran™, Premium, GE Healthcare Life Science, Munich, Germany). The membrane was blocked in 1× Tris-buffered solution with 0.1% Tween-20 (0.1% TBST) containing 5% BSA for 1 h at 4 °C with shaking, and then incubated with primary antibody in diluted blocking buffer at 4 °C overnight with shaking. To confirm equal protein loading, the blots were stripped and reprobed with anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody, anti-EGFR antibody, or anti-ERK1/2 antibody. Bands were detected using the ChemiDoc™ MP Imaging System (Bio-Rad, Hercules, CA, USA). Densities of the bands were measured using ImageJ software (version 1.48, NIH). All experiments were performed in triplicate. The following antibodies were used: goat anti-P2Y2R (1:400; sc-15209, Santa Cruz Biotechnology, Inc.), rabbit anti-EGFR (1:200; sc-03, Santa Cruz Biotechnology, Inc.), mouse anti-ERK1/2 (1:2000; #4696, Cell Signaling Technology, Danvers, MA, USA), mouse anti-Akt (1:2000; #2920, Cell Signaling Technology), rabbit anti-phospho-EGFR (1:2000; #2234, Cell Signaling Technology), rabbit anti-phospho-ERK (1:2000; #4370, Cell Signaling Technology), rabbit anti-phosphor-Akt (1:2000; #4060, Cell Signaling Technology), and rabbit anti-GAPDH (1:400; ab8245, Abcam, Cambridge, MA, USA).

2.2.5. Cell migration assay

In vitro cell migration assays were performed using the Oris™ 96-well 2D assay kit (Platypus Technologies, LLC, Madison, WI, USA) according to the manufacturer's protocol. This kit utilizes a silicone stopper to cover a 2-mm-diameter zone and to restrict cell seeding to the outer annular regions of the wells. Removal of the stoppers reveals a 2-mm-diameter unseeded gap in the center of each well into which the seeded cells may then migrate into during each treatment. Unlike a scratch wound assay, this protocol prevents the extracellular release of ATP and byproducts from damaged cells. Briefly, suspended cells (5×10^5 cells/mL) in 100 μL KSFM were seeded in each well of a 96-well plate and incubated in a humidified chamber (37 °C, 5% CO_2) for 18 h. All stoppers sealing the central zone were removed and wells were gently washed with PBS. After washing, fresh KSFM with various treatments was added to each well. Cells were then incubated in a humidified incubator (37 °C, 5% CO_2) and progression of cell migration was monitored. After an additional 24-h incubation, cells were stained with 1% toluidine blue, and images were taken using a digital charge-coupled device (CCD; MC170-HD, Leica Microsystems) mounted on a light microscope (CKX-41, Olympus). The percentage of gap closure was calculated using ImageJ software (version 1.48, NIH).

2.3. Statistical analysis

Analysis of variance (ANOVA) was used to analyze differences between groups. Differences were considered significant when $P < 0.05$. Mean values and SEM values are presented.

3. Results

3.1. In vivo animal experiments

3.1.1. Diquafosol promotes corneal epithelial wound healing in rats

After creating a 3-mm diameter epithelial defect on rat corneas as described in Section 2.1.1, we compared the wound closure in control eyes and diquafosol-treated eyes over 36 h (Fig. 1). The diquafosol-treated eyes showed smaller defect sizes than controls at 12 h and 24 h after treatment. At 36 h after diquafosol treatment,

epithelial defects were closed in all eyes, although surface irregularity and punctate erosions were observed in some eyes. On the other hand, control eyes showed more coarse and irregular surface with punctate erosions, and epithelial defect remained in 50% of control eyes (3/6) at 36 h. On image analysis, the percentages of wound closure (%) in diquafosol-treated eyes were significantly higher at 12 and 24 h (63.4 ± 2.0% and 98.1 ± 1.1%, respectively) than in control eyes (42.7 ± 2.5% and 82.3 ± 3.2%, respectively). These results showed that topical diquafosol accelerates corneal epithelial wound healing.

3.1.2. *P2Y2R is dominantly stained in the wound margin of corneal epithelium*

We also observed the expression pattern of P2Y2R in wounded and unwounded corneal epithelium by immunofluorescence staining (Fig. 2). P2Y2R was weakly stained in the normal epithelium of unwounded corneas and in the healed multilayered epithelium of diquafosol-treated corneas and control corneas, whereas it was strongly stained in the epithelial margin around the wound defect in diquafosol-treated corneas and control corneas. There was no hypertrophy or abnormal polarity of healed epithelium in diquafosol-treated corneas when wound closure was complete (Appendix).

3.2. *In vitro experiments*

3.2.1. *Diquafosol promotes THCE cell proliferation*

We performed cell proliferation/cytotoxicity assays in THCE cells with various concentrations of diquafosol to determine the range of effective or toxic concentrations *in vitro*. The absorbance values were measured at 30 min, 1, 2, 4, 8, 12, 24, and 48 h (Fig. 3). The absorbance values of wells treated with 20, 50, 100, and 200 μM diquafosol (0.605 ± 0.011, 0.632 ± 0.011, 0.602 ± 0.012, and 0.627 ± 0.044 at 24 h, and 0.643 ± 0.034, 0.677 ± 0.045, 0.681 ± 0.043, and 0.658 ± 0.026 at 48 h, respectively) were significantly higher than those of KSFM-only wells (0.473 ± 0.030 and 0.484 ± 0.007 at 24 and 48 h, respectively, all $P < 0.05$), and were not lower than those of EGF-treated wells (0.658 ± 0.013 and 0.717 ± 0.013 at 24 and 48 h, respectively, all $P < 0.05$). On the other hand, diquafosol at a concentration higher than 2000 μM significantly inhibited cell proliferation 12 h after incubation, compared to KSFM only.

3.2.2. *Diquafosol elicits $[Ca^{2+}]i$ elevation in THCE cells*

We measured the fluorescence intensity of serial images at 1.0-s intervals for 5 min to determine the change of $[Ca^{2+}]i$ in diquafosol-stimulated cells (Fig. 4). Diquafosol-stimulated cells in KSFM showed a rapid increase in the fluorescence intensity at 10 s post-stimulation, a peak of intensity at around 30 s post-stimulation, and a subsequent slow decrease to baseline (Fig. 4A). The relative increase of fluorescence intensity ($\Delta F/F_0$, %) reached to the maximum value (32.5%) at 38.889 s (about 28 s post-stimulation) in KSFM. Diquafosol-stimulated cells in calcium-free KSFM showed the same pattern of response as those in KSFM, although the maximum value (23.0% at 35 s post-stimulation) was slightly lower in the former. Suramin pretreatment inhibited $[Ca^{2+}]i$ elevation in diquafosol-

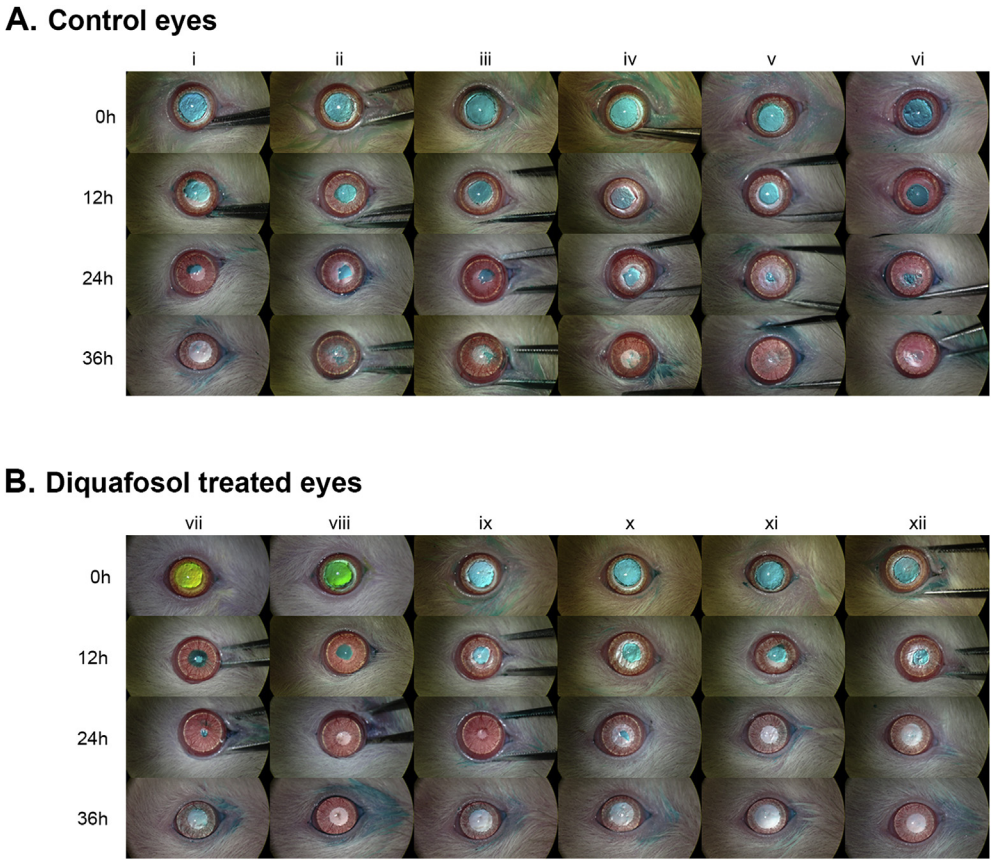

Fig. 1. The effect of topical diquafosol on epithelial wound healing of rat corneas. A 3-mm-diameter epithelial defect was produced at the center of the corneas, and vehicle (A, n = 6) or diquafosol (B, n = 6) was applied topically four times a day. Images of epithelial defects stained with lissamine green dye were obtained at 0, 12, 24, and 36 h after wounding. The size of the remaining epithelial defects was smaller in diquafosol-treated eyes (B) at 12 and 24 h than in control eyes (A) at 12 and 24 h, respectively. At 36 h, no epithelial defects were not found in diquafosol-treated eyes (B), whereas small defects were found in some control eyes (A).

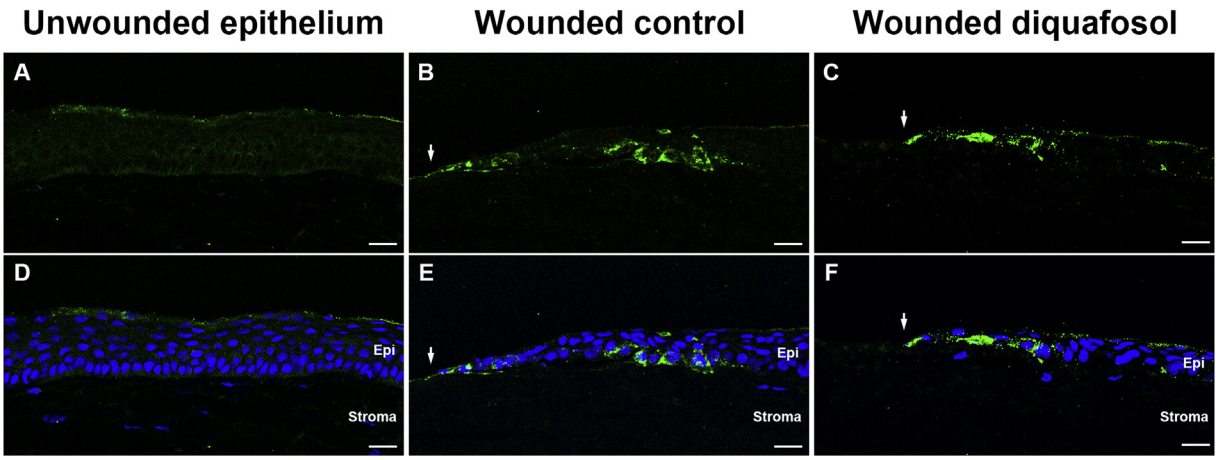

Fig. 2. The immunofluorescence expression pattern of P2Y2R in unwounded and wounded epithelium of rat corneas. The immunostaining pattern of P2Y2R (green) were observed in the unwounded epithelium of healthy corneas (A), the wounded epithelium of control corneas (B) and diquafosol-treated corneas (C) with DAPI (blue) nuclear counterstaining (D, E, and F, respectively). P2Y2R was weakly stained in the unwounded epithelium of healthy corneas (A, D). In contrast, P2Y2R was strongly stained in the re-epithelialized margin (white arrow) of the wounded epithelium of control corneas (B, E) and diquafosol-treated corneas (C, F). Scale bars = 20 μm.

stimulated cells in both KSFM and calcium-free KSFM.

3.2.3. *EGFR/ERK signaling is involved in diquafosol-induced epithelial healing*

We evaluated whether diquafosol promotes wound closure of THCE cell monolayers and activates EGFR, ERK, and Akt signaling pathways during wound closure. In the cell proliferation assay described in Section 2.2.2, we found that 20–200 μM diquafosol accelerated cell proliferation in THCE cells. Thus, 100 μM diquafosol was used to perform western blot analysis and wound closure assays. At 2 min after incubation with diquafosol, phosphorylation of EGFR was observed and persisted until 10 min. ERK phosphorylation was found from 5 to 30 min after incubation with diquafosol (Fig. 5A). Phosphorylation of Akt, however, was not affected by diquafosol stimulation. Phosphorylation of ERK by diquafosol was not observed in cells pretreated for 30 min with suramin or BAPTA/AM but was observed, albeit reduced, in cells pretreated with AG1478, an EGFR inhibitor (Fig. 5B). The results of the *in vitro* cell migration assay corresponded to these expression patterns in western blot analysis (Fig. 5C). The percentage of gap closure in diquafosol-treated cells (36.1 ± 2.0%) was significantly higher than that in non-treated cells (26.9 ± 2.0%, $P < 0.05$). The promotion of gap closure by diquafosol was inhibited in cells pretreated with suramin, BAPTA/AM, AG1478, and U0126 (percentage of gap closure:

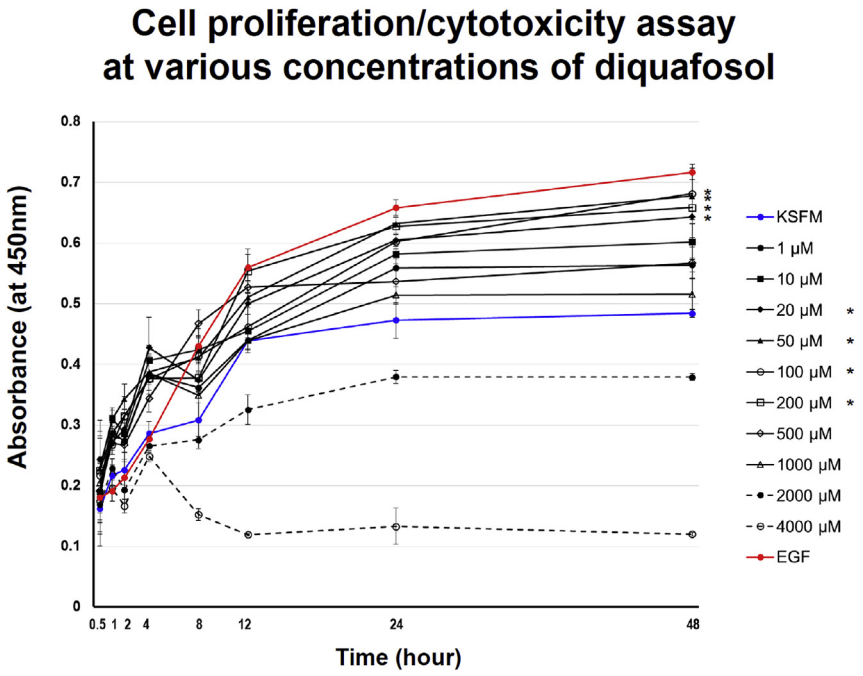

Fig. 3. The effect of diquafosol on cell proliferation *in vitro*. THCE cells (1×10^4 cells per well) were seeded into 96-well plates and starved for 12 h. Cells were then incubated at various concentrations of diquafosol and cell proliferation was assayed using WST-8. Absorbance values at 450 nm are given on the y-axis. After 24 h, the absorbance values were significantly higher at 20, 50, 100, and 200 μM, compared to control with only KSFM (*$P < 0.05$). Diquafosol promoted cell proliferation at concentrations from 20 to 200 μM, but inhibited cell proliferation at concentrations above 2000 μM.

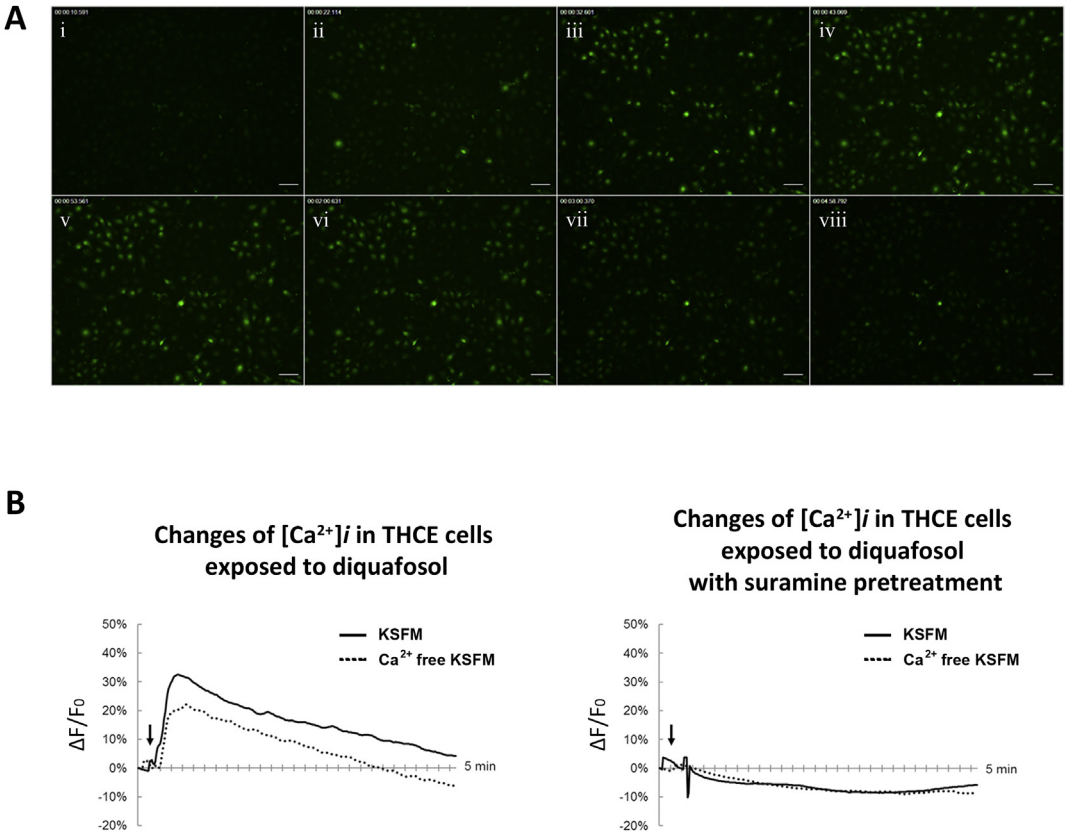

Fig. 4. Changes of $[Ca^{2+}]i$ in THCE cells exposed to diquafosol. (A) After diquafosol was loaded to Fluo-4/AM-pretreated THCE cell monolayers, fluorescence images at 516 nm were sequentially obtained at an interval of 1.0 s for 5 min. Diquafosol was loaded at 10 s after image recoding was started (i). The consecutive images indicate the following time points; ii; 22 s, iii; 32 s, iv; 43 s, v; 53 s, vi; 2 min, vii; 3 min, viii; 5 min after image recording. Fluorescence intensity (F) for each image was measured using ImageJ software, and the relative change of fluorescence intensity $[(F - F_0)/F_0]$ was calculated to express the changes of $[Ca^{2+}]i$. $[Ca^{2+}]i$ increased rapidly and reached its peak within 40 s from the time diquafosol was loaded (black arrows) in the presence (solid line) and in the absence (dotted line) of calcium ion in KSFM (B, left panel). Suramin pretreatment (200 μM) abolished diquafosol-induced $[Ca^{2+}]i$ elevation in the presence and in the absence of calcium ion in KSFM (B, right panel). KSFM, keratinocyte serum-free medium; F, fluorescence intensity at each time point; F_0, initial fluorescence intensity. Scale bar = 100 μm.

7.6 ± 0.9%, 8.7 ± 1.9%, 20.8 ± 2.0%, and 10.2 ± 2.0%, respectively, all $P < 0.05$).

4. Discussion

The effect of diquafosol on epithelial healing has not been reported hitherto, although many previous studies have reported the effects of diquafosol on tear film and the clinical parameters related to dry eye syndrome (Arita et al., 2013; Jumblatt and Jumblatt, 1998; Kaido et al., 2013; Kamiya et al., 2012; Koh et al., 2013, 2014; Kojima et al., 2014; Matsumoto et al., 2012; Mori et al., 2014; Nakamura et al., 2012; Shimazaki-Den et al., 2013; Takamura et al., 2012; Tauber et al., 2004; Toda et al., 2014; Yokoi et al., 2014). Here, we observed that diquafosol accelerates corneal epithelial healing in experimental rats. There are two possible explanations for this. First, diquafosol-induced tear film stability may facilitate epithelial healing. Second, diquafosol seems to play a role in corneal epithelial cell proliferation and migration.

As for the first mode of action, diquafosol is well known for stabilizing the tear film on the ocular surface through fluid efflux from the conjunctival epithelium (Li et al., 2001; Murakami et al., 2004), secretion of soluble mucin by conjunctival goblet cells (Murakami et al., 2003), and secretion of membrane-associated mucin by the corneal epithelium (Fujihara et al., 2002). Tears normally contain growth factors that facilitate epithelial homeostasis (Klenkler et al., 2007). However, there is no evidence that diquafosol stimulates tear production. Furthermore, two or three days of wound closure is too short a period for the tear film to be significantly affected by diquafosol.

Regarding the second mode of action, an effect of diquafosol on corneal epithelial healing is expected based on previous evidence. Diquafosol is a dinucleotide derivative of UTP, and has P2Y2R agonist activity similar to that of UTP. In rabbit corneas, UTP promotes epithelial wound healing (Pintor et al., 2004). The ATP released from damaged cells also plays a critical role in the epithelial wound healing process (Yin et al., 2007). Nucleotides including ATP and UTP stimulate epithelial cell migration and proliferation via P2YR-induced downstream signaling events including Ca^{2+} elevation, and the phosphorylation of EGFR and ERK (Boucher et al., 2010; Crooke et al., 2008; Mediero et al., 2008; Muscella et al., 2004). In the present study, we found that diquafosol stimulated cell proliferation and migration through the $[Ca^{2+}]i$—EGFR/ERK signaling pathway in the same manner as extracellular nucleotides.

Through *in vitro* experiments using THCE cells, we confirmed that cell proliferation was accelerated when we applied 20–200 μM diquafosol over 48 h $[Ca^{2+}]i$ was immediately elevated in diquafosol-stimulated cells. Western blot analysis and cell migration assay revealed that 100 μM diquafosol induced EGFR phosphorylation at 2 min post-stimulation, followed by ERK phosphorylation at 5 min post-stimulation. This ERK phosphorylation was attenuated by a P2Y2R antagonist, EGFR inhibitor, and

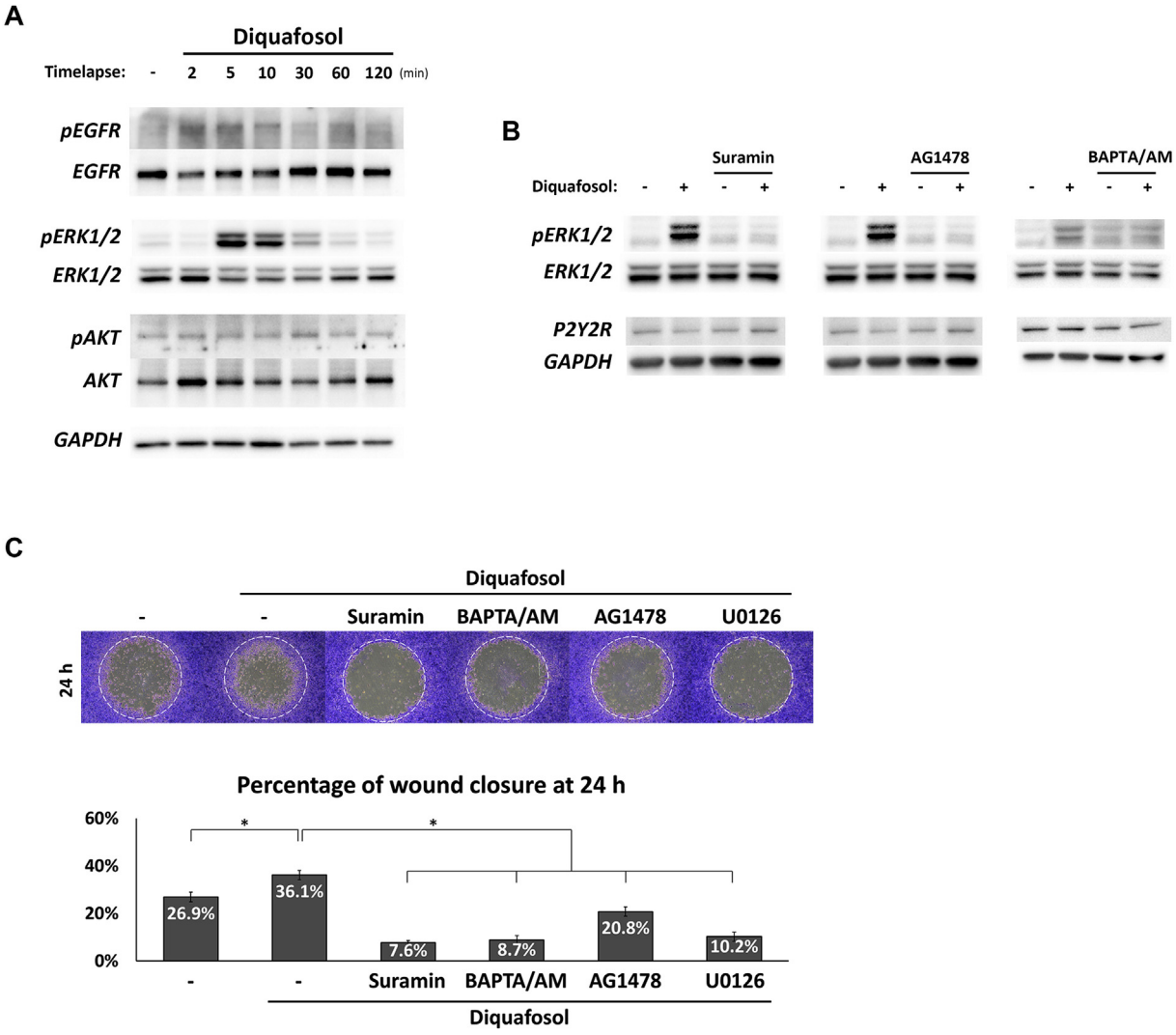

Fig. 5. Diquafosol activates EGFR/ERK signaling, which is $[Ca^{2+}]i$ dependent in THCE cells. (A) The phosphorylation of EGFR, ERK, and Akt was assessed in THCE cells at indicated times after diquafosol incubation by western blot analysis. (B) Serum- and growth factor-starved THCE cells were treated with 200 μM suramin, 5 μM AG1478, or 5 μM BAPTA/AM for 30 min and further incubated with or without 100 μM diquafosol for 5 min. Cell lysates were immunoblotted with phospho-ERK1/2, ERK1/2, phospho-Akt, Akt, and P2Y2 antibody. GAPDH was used as a probe to normalize protein loading. The results are representative of three independent experiments. (C) Cell migration assay for 24 h was performed to confirm the result of western blot. The results are representative of three independent experiments. $^*P < 0.05$.

intracellular calcium chelator. These results indicate that diquafosol accelerates corneal epithelial healing through $[Ca^{2+}]i$-mediated EGFR/ERK signaling pathway by activating P2Y2R, in the same manner as extracellular nucleotides.

Diquafosol induced ERK phosphorylation and promoted cell migration *in vitro* when EGFR was blocked by AG1478, although the response was reduced. This indicated the existence of an EGFR-independent signaling pathway through which diquafosol stimulates ERK. Other studies have shown that ERK phosphorylation may occur independent of EGFR (Andreev et al., 2001; Boucher et al., 2007; Montiel et al., 2006). In an EGFR-independent pathway, P2Y2R initiates ERK activation by involving Shc and Grb2, or RAFTK and PKC via $[Ca^{2+}]i$ elevation (Soltoff et al., 1998). This result can be explained as an alternate possibility supported by studies that have shown that differential phosphorylation of specific EGFR tyrosine residues, including 845, 1068, 1086, and 1173, occurs in response to specific biologic activation (Moro et al., 2002).

In EGFR-dependent or -independent pathways, P2Y2R-mediated ERK activation is calcium-dependent (Chuderland and Seger, 2008). Our data also showed that intracellular calcium chelators potently inhibited diquafosol-induced ERK phosphorylation as well as cellular migration. P2Y2R, a G protein-coupled receptor, regulates mobilization of $[Ca^{2+}]i$ from internal storage through phospholipase C activation and production of inositol triphosphate (IP3) and diacylglycerol (DAG) (Neary et al., 1999). Previous studies have reported that P2Y2-mediated $[Ca^{2+}]i$ elevation was not affected by the presence or absence of extracellular calcium, and that it was inhibited by depletion of internal calcium storage or blockage of second messengers (Green et al., 1997; Tovell and Sanderson, 2008; Viana et al., 1998). In our study, diquafosol-induced $[Ca^{2+}]i$ elevation was blocked by suramin, a P2Y2R antagonist, adding evidence that $[Ca^{2+}]i$ elevation is triggered by diquafosol acting on P2Y2R. The diquafosol-induced calcium response was the same in the presence and absence of calcium in media, although the peak intensity was slightly lower in calcium-free KSFM, indicating that $[Ca^{2+}]i$ was mobilized from internal storage. We thought that the slightly reduced peak intensity was probably due to the 30-min incubation in calcium-free KSFM prior to diquafosol stimulation.

In summary, we demonstrated that topical diquafosol, a P2Y2R

agonist, promotes corneal epithelial wound healing *in vivo*. This was supported by *in vitro* evidence that diquafosol induces cell proliferation and migration through $[Ca^{2+}]i$-dependent ERK activation. We suggest that diquafosol may be helpful in treating ocular surface disease with delayed healing, including desiccated ocular surface in dry eye disease. Wound healing, however, is a complicated process regulated by various factors including inflammation, adhesion molecules, and extracellular matrix, besides proliferation and migration. Therefore, further studies are needed to explore the role of extracellular nucleotides and purinergic receptors in the wound healing process.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.exer.2015.10.013.

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