Department of Ophthalmology, the People's Hospital of Binzhou City, Binzhou, People's Republic of China

Effect of lithium chloride on endoplasmic reticulum stress-related PERK/ROCK signaling in a rat model of glaucoma

XIAO-BO SUN, HONG-E LU, YUAN CHEN, XIAO-HUI FAN, BIN TONG

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Xiao-bo Sun, Department of Ophthalmology, the People's Hospital of Binzhou City, Binzhou, Shandong Province 256610, People's Republic of China

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Elevated intraocular pressure (IOP) is considered as the major risk factor for the loss of retinal ganglion cells (RGCs) and their axons in glaucoma. Lithium chloride (LiCl) inhibits glycogen synthase kinase-3 beta (GSK-3β) and attends PERK-induced endoplasmic reticulum stress (ERs) transition. PERK is a type I transmembrane protein located in the endoplasmic reticulum. PERK pathway activation takes place in ERs early inhibiting protein synthnesis to protect cell and promote cell survival. Here, we firstly evaluate that LiCl reduced IOP when administered intraperitoneally. After 6 weeks, IOP dropped by around 21.9% in LiCl treated rats. Then we investigated the effects of LiCl on PERK-mediated signaling pathways. LiCl treatment activated PERK and inhibited the expression of ROCK-1and ROCK-2 in a rat model of glaucoma. Collectively, these results suggest that LiCl reduced the IOP through the phosphorylation of PERK by the regulation of PERK/ROCK signaling in glaucoma rat model.

1. Introduction

The glaucomas are a family of diseases of the eye that can be characterised by a progressive retinal ganglion cell (RGC) loss (Wheeler and Woldemussie 2001). It is projected to affect 79.6 million people by 2020 (Gupta and Yücel 2007). Elevated intraocular optical pressure (IOP) is a major risk factor for glaucoma (Caprioli and Coleman 2008). IOP reduction is the therapy most commonly employed in clinical practice to reduce the risk of glaucoma progression.

Glycogen synthase kinase-3 beta (GSK-3β), which is expressed specifically in the central nervous system (Dill et al. 2008), is a pleiotropic enzyme which is negatively-regulated by several intracellular signaling pathways and has roles in cell growth, proliferation, survival, cytoskeletal stability and microtubule dynamics (Nishino et al. 2008; Chung et al. 2014).

Lithium is a drug that is used as a mood stabilizer for the treatment of bipolar disorders. The drug elicits neuroprotective effects when used to treat various neural diseases including stroke, Parkinson's disease, and spinal cord injury (Young 2009). In recent years a number of studies have shown that lithium chloride (LiCl), which is a specific GSK-3β inhibitor, can improve the symptoms of neurodegenerative diseases. Recent clinical experiments prove that LiCl can inhibit the progression of amyotrophic lateral sclerosis and inactivation of GSK-3β can result in the prevention or in apoptotic injury in neurons (Meyer et al. 2010). It has been reported that LiCl contributes to neuroprotection for retinal neuronal injury in the rat (Zhuang et al. 2009). However, the underlying mechanism is not clear. This study provides new insights into the protective mechanisms of lithium chloride to the neuronal system.

2. Investigations and results

2.1. Effects of LiCl treatment on intraocular pressure

The mean basal intraocular pressure (IOP) of the control right eye during the 6 weeks of this study was 15.8 ± 1.9 mmHg. Episcleral vein cauterization (EVC) of the experimental left eye resulted in a significant increase in IOP ($p < 0.01$), leading to a mean IOP of 24.2 ± 4.1 mmHg (Fig. 1A). Treatment with intraperitoneal PBS did not induce significant changes in IOP in EVC eyes with respect to EVC eyes which were not further treated. Daily application of LiCl produced a significant IOP reduction in EVC eyes. Thus, the mean IOP of these eyes following glaucoma induction was reduced to normal values following LiCl treatment (24.2 ± 4.1 mmHg vs.18.9 ± 3.4 mmHg, $p < 0.001$) (Fig. 1B).

2.2. Activation of PERK and inactivation of CHOP and ATF6 following LiCl treatment

We first screened, by RT-PCR, the expression of mRNA in rat retina. We specifically analyzed the expression of: CHOP, ATF6 and PERK. The result showed that the expression of CHOP and ATF6 were not markedly changed in all rats, suggesting that the two genes were not involved in the LiC1 treatment (Fig. 2B and 2C). The mRNA levels showed that gene expression of PERK was lightly increased in LiCl-treated rats but without significant difference.

We next examine the protein expression of PERK. As shown in Fig. 2D, treatment with LiCl significantly increased the level of PERK, a marker of ER stress, by 66% compared to the untreated controls ($P < 0.01$).

2.3. Regulation of the PERK/ROCK pathway

In order to explore whether the glaucoma protecting effect of LiCl was regulated by PERK/ROCK pathway, we next investigated the protein changes of p-PERK, ROCK-1, ROCK-2 by western blot. RT-PCR was also applied to detect the transcript of ROCK-1and ROCK-2. Compared to the model group, the

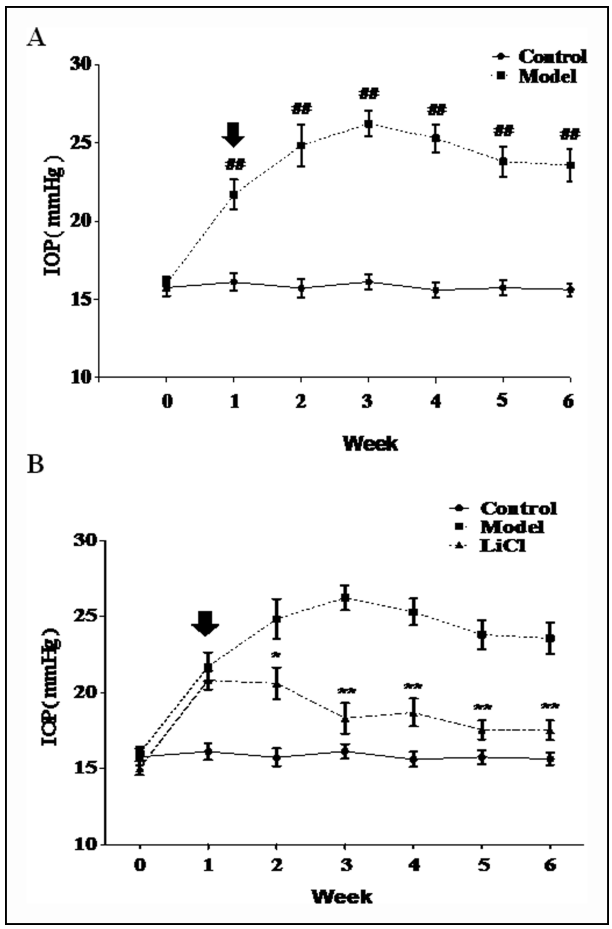

Fig. 1: Changes in intraocular pressure (IOP) following different anti-glaucoma treatments. (A) Control animals treated daily with topical PBS and weekly with an intraperitoneal PBS injection; right eye, control; left eye, episcleral vein cauterization (EVC). (B) Animals were treated weekly with an intraperitoneal injection of LiCl. Arrows indicate the beginning of the treatment. (n = 15 per group, data were present as mean ± SEM, $^{\#\#}p < 0.01$ compared with control, $^{*}p < 0.05$ $^{**}p < 0.001$ compared with model).

phosphorylation pattern of PERK was increased by 32% in the LiCl-treated group. These results also suggested that protein expression of ROCK-1, ROCK-2 in the LiCl-treated group was significantly decreased (Fig. 4B and 4C).

3. Discussion

In this study, we demonstrated the glaucoma protection effect of LiCl. This observation is supported by the results obtained from IOP measurements. Compared to the model group, the mRNA expression level of PERK increased as well as the protein phosphorylation of PERK. The mRNA and protein levels of ROCK-1, ROCK-2 were also notably increased, however, there were no significant differences in CHOP and ATF6 gene and protein expression.

The endoplasmic reticulum (ER) signal transduction pathway is one of the three classic apoptosis pathways. It is the site for protein folding, assembly and transportation. When cells suffer from different intensity of stimulation such as oxidative stress, unfolded or misfolded protein will accumulate in the endoplasmic reticulum. The unfolded protein response (UPR) is an ER-mediated response to the accumulation of large amounts of unfolded or misfolded proteins in the ER. The UPR is mediated by the sequential and concerted activation of three key players, namely, PERK, ATF6, and CHOP. In the present study, the change of PERK, phosphor-PERK, ROCK-1, ROCK-2 in rat retina mRNA and protein suggested the PERK/ROCK signaling to be involved in the LiC1 mediated protection against glaucoma. In this paper, we found that the ativation of PERK played an major role in mediating ERs after LiCl treatment. PERK is also suggested to contribute to cell death during prolonged and severe ER stress.

ROCK, a Rho-associated serine/threonine kinase, has been characterized as an effector of Rho. The vasodilating effect of ROCK inhibitors, as described above, has also been documented in the retina (Tan et al. 2012). Though the precise role of the Rho-ROCK pathway in glaucoma pathology remains unknown, elevated levels of optic nerve head (ONH) RhoA have been reported in glaucomatous eyes (Inoue and Tanihara 2013). ROCK inhibitors can protect neurons against various stresses and promote regeneration of crushed retinal ganglion cell axons (Allaire and Dumais 2012). ROCK inhibitors may also slow down the progression of glaucomatous optic neuropathy by working directly on the optic disc blood vessels. Whitlock et al. (2009) examined the effects of ROCK inhibitors by using both pharmacological and genetic approaches in mice. The ROCK inhibitors, Y-27632 and Y-39983, significantly lowered IOP more than latanoprost, a commonly used anti-glaucoma agent. In addition, mice deficient in either ROCK-1 or ROCK-2 were created, and these mice had significantly lower IOP than their wild-type littermates. These data indicate that both pharmacological and genetic ROCK inhibition can lower IOP (Inoue and Tanihara 2013).

In conclusion, ERs is associated with neuronal cell death in diabetic retinas and retinas incubated in high glucose medium (Zhong et al. 2012). The neuroprotective effect of LiCl is correlated with the suppression of ERs-related factors, PERK expression. In this paper, we found that the IOP was significantly decreased associated with an increae in PERK. Further *in vivo* studies examined the molecular link between p-PERK and ROCK. We demonstrated, for the first time, that one of the mechanisms of LiCl neuroprotection might involve inhibition of the phosphorylation of PERK through down-regulation of ERs-ROCK. We set out to determine the effect of LiCl on the glaucoma and molecular pathways mediating these changes. This information could be useful in the consideration of clinical applications of LiCl in glaucoma filtration surgery and for understanding the mechanism of surgery induced scar formation.

4. Experimental

4.1. Materials

Lithium chloride was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Antibodies specific for PERK (sc-9477), p-PERK (sc-32577), ROCK-1 (sc-374388), ROCK-2 (sc-1851) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), antibody specific for ATF6 (ab37149) was purchased from (Abcam, MA, USA). CHOP (#2895) was obtained from Cell Signaling Technology (Beverly, MA, USA).

4.2. Animals

Thirty adult male Sprague–Dawley (SD) rats, weighing 200–250 g, were provided by Shanghai Slac Laboratory Animal Ltd (Shanghai, China). Rats were housed with free access to food and water under a natural day/night cycle. Rats were acclimated for 7 days before any experimental procedures. All experimental protocols were in accordance with the Institutional Animal Care and Use Committee of Jiangsu Provincial Institute of Materia Medica.

4.3. Induction of glaucoma and IOP measurement

The animals were anesthetized by intraperitoneal injection of ketamine (50 mg/kg) plus xylazine (5 mg/kg), and local anesthetic drops were applied to the eye. IOP was elevated by cauterizing three episcleral veins of the left

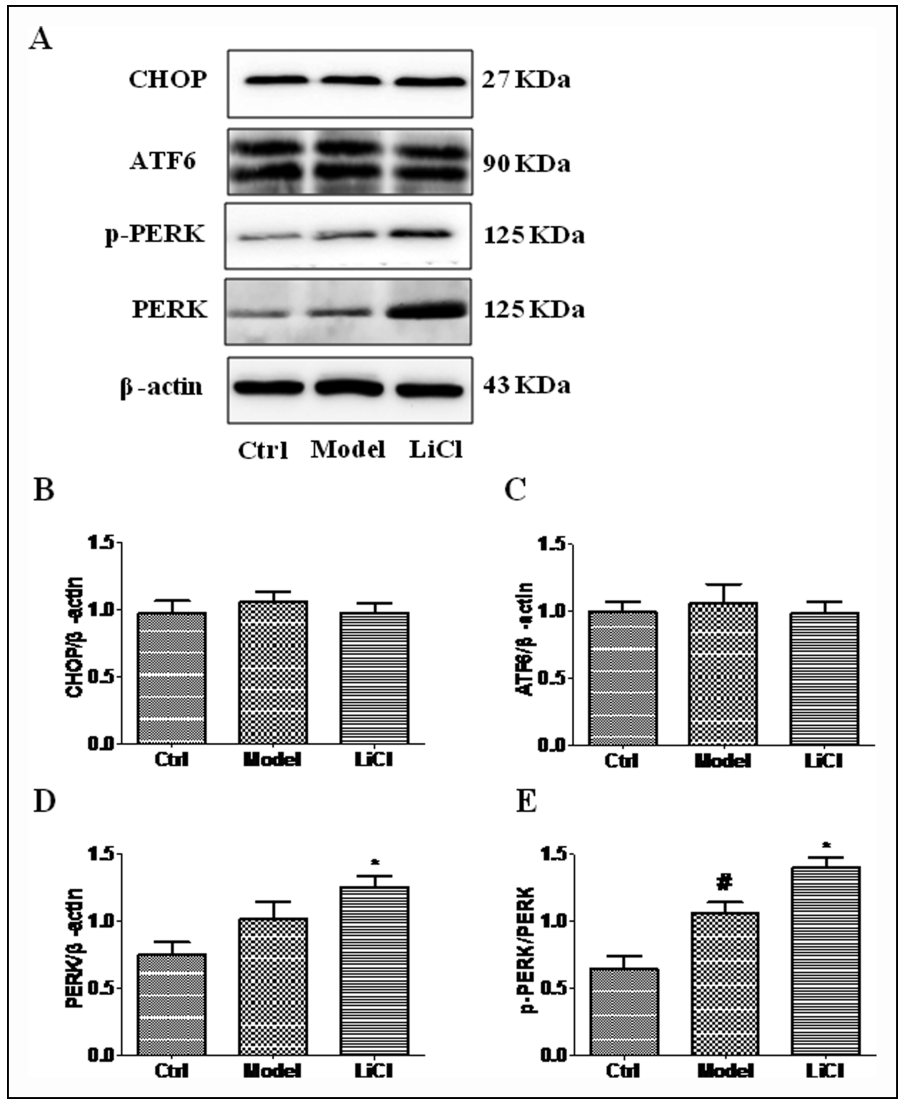

Fig. 2: The protein levels of CHOP, ATF6, PERK and p-PERK in rat retina after 5 weeks treated. (A) Chop, ATF6, PERK and p-PERK protein level was detected by Western Blot. (B)(C)(D)(E) Data analysis was measured by gray scale scanning (n = 3 per group, data were present as mean ± SEM, $^{\#}p < 0.05$ compared with control, $^{*}p < 0.05$ compared with model).

eye, as has been described previously (Hernández et al. 2008). The right eyes of all groups were not operated in order to be considered as control eyes. This procedure resulted in blockage of the venous outflow, which represents 50% of the total area available for episcleral venous return. IOP was measured weekly with an applanation tonometer (TonoPen XL, Mentor, Norwell, MA) while rats were in awake at the same time of day (3 p.m), because it has been previously demonstrated that anesthetics cause a reduction in IOP (Urcola et al. 2006).

4.4. Pharmacological treatments

Three days after surgery, when the IOP was elevated, animals were treated and distributed into different groups: (I) control rats (n = 15) were treated with 10 mM PBS (7 microliters per gram of body weight) with a daily peritoneal injection; (II) fifteen rats received i.p. injections of either 0.6 M LiCl (7 microliters per gram of body weight) daily for 5 weeks (Noble et al. 2005).

4.5. Quantification of mRNA expression

The gene expression levels of CHOP, ATF-6, PERK and ROCK in the left eyes were determined by real-time quantitative reverse-transcription polymerase chain reaction with the use of ABI 7700 and specific primers as reported previously (Feng et al. 2011; Medigeshi et al. 2007; Lin et al. 2013; Saito et al. 2010). β-Actin forward 5'-GGG-AAA-TCG-TGC-GTG-ACA-T-3', reverse 5'-CAG-GAG-GAG-CAA-TGA-TCT-T-3'. CHOP ward 5'- GGAAAGTG-GCACAGCTTGCT -3', reverse 5'- CTGGTCAGGCGCTCGATT -3'. ATF-6 forward 5'-GGATTTGATGCCTTGGGAGTCAGAC-3', reverse 5'-ATTTTTTTCTTTGGAGTCAGTCCAT-3'. PERK forward 5'-AAGAT GGTACAGTGGACGGC-3', reverse 5'-CCGTGTTCCTGGTGAAATCT-3'. ROCK forward 5'-GATTGGATTTTCCTGCCTTAAGA-3', reverse 5'-CCTGGTGGATTTATGCCTTACC-3'.

4.6. Western blot analysis

Retina samples were processed for Western blot analysis. Equal amounts (50 μg) of protein were subjected to 8% and 10% SDS-PAGE gel, and transferred onto a PVDF membrane in a semi-dry system (Bio-Rad, USA). The membranes were blocked with 5% BSA in Tris-buffered saline containing 0.1% Tween 20, and incubated with specific antibodies. The signals were developed using Super-Signal West Pico chemiluminescent substrate (Millipore, Watford, USA) and visualized with Quantity One software 4.6.9.

4.7. Statistical analysis

The results are presented as mean ± SEM. All data were statistically analyzed with SPSS 11.0 statistical package for Windows version. The means among groups were compared using one-way ANOVA, followed by Student–Newman–Keuls's *post hoc* test. Statistical significance was set at $P < 0.05$.

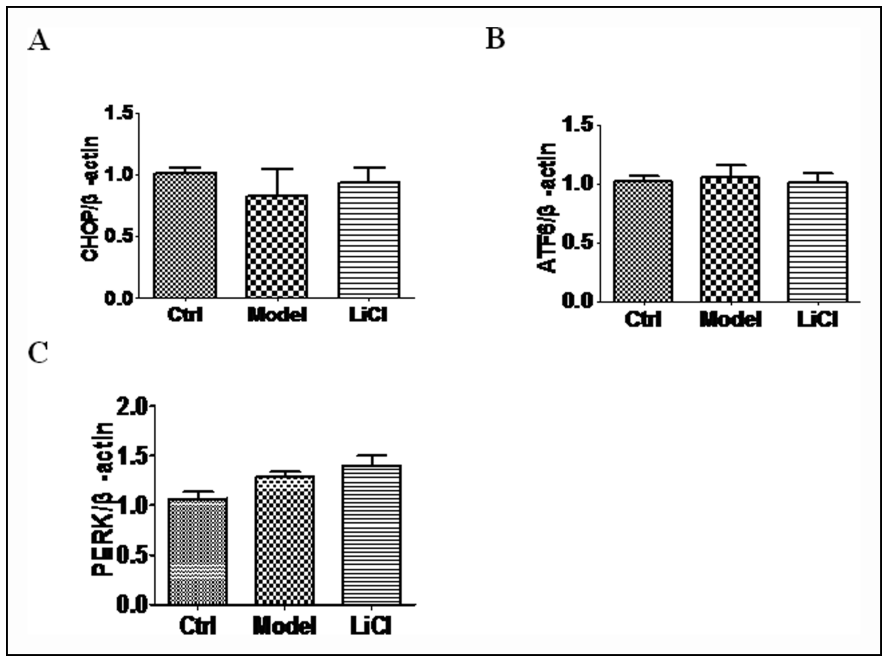

Fig. 3: The mRNA levels of ROCK-1 (A) and ROCK-2 (B) in rat retina after 5 weeks treated was detected by qPCR. The data shows significant difference between each group (n = 3 per group, data were present as mean ± SEM).

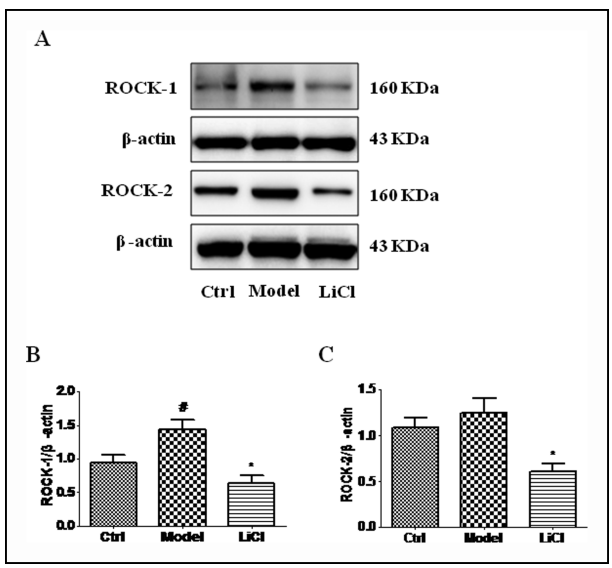

Fig. 4: The protein levels of ROCK-1 and ROCK-2 in rat retina after 5 weeks treated. (A) ROCK-1 and ROCK-2 protein level was detected by Western Blot. (B)(C) Data analysis was measured by gray scale scanning (n = 3 per group, data were present as mean ± SEM, $^{*}p < 0.05$ compared with model).

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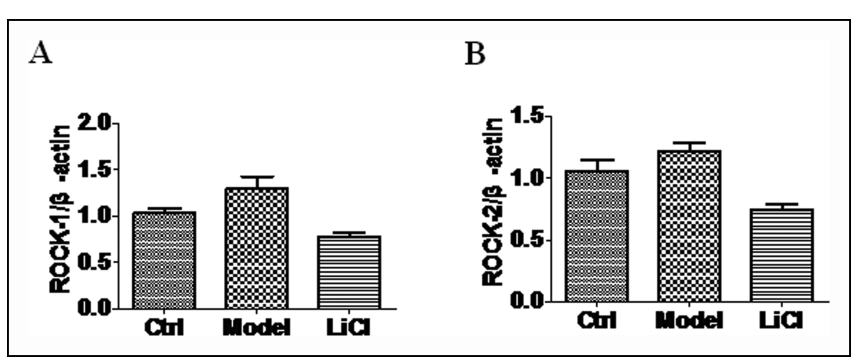

Fig. 5: The mRNA levels of ROCK-1(A) and ROCK-2(B) in rat retina after 5 weeks treated was detected by qPCR. The data shows significant difference between model group and LiCl-treated group (n = 3 per group, data were present as mean ± SEM, $^{*}p < 0.05$ compared with model).

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