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Aquaporin 5 increases keratinocyte-derived chemokine expression and NF-κB activity through ERK activation

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ABSTRACT

Aquaporin-5 (AQP5) is a water-selective channel protein that is expressed in submucosal glands and alveolar epithelial cells in the lungs. Recent studies have revealed that AQPs regulate not only water metabolism, but also some cellular functions such as cell growth and migration. Here, we report the role of AQP5 in inflammatory responses. In MLE-12 cells, knockdown of AQP5 using siRNA (10–50 nM) attenuated TNF-α-induced expression of keratinocyte chemoattractant (KC) mRNA and protein. Conversely, in NIH-3T3 cells, overexpression of AQP5 increased KC expression, NF-κB activation, and ERK phosphorylation. The AQP5-induced increase of KC expression was diminished by treatment with ERK inhibitors. Taken together, we propose a new function of AQP5 as an inflammatory signal potentiator, which may be mediated by increased activation of ERK and NF-κB.

1. Introduction

Aquaporins (AQPs) are a family of transmembrane channels that allow rapid movement of water and, in some cases, small molecules across the plasma membrane [1,2]. Currently, 13 mammalian AQPs (AQP0–12) have been identified [3]. Among them, AQP5 is selectively expressed in the apical plasma membrane of various secretory glands, such as the airway submucosal glands, and alveolar type I epithelial cells in the lungs [4,5]. Targeted deletion of AQP5 in mice results in considerable reduction of fluid secretion from submucosal glands [6] and a 10-fold decrease of osmotic water permeability of the alveolar-capillary barrier in distal lungs [7]. These previous findings suggest that AQP5 plays a critical role in maintenance of normal lung water homeostasis, and changes in the amount of cell surface expression of AQP and/or its function may contribute to abnormal water metabolism in various lung diseases such as lung edema.

A recent study has indicated that AQPs regulate not only water metabolism, but also various cellular functions such as cell migration, proliferation, and invasion. For example, AQP3 expression in keratinocytes has been shown to facilitate migration in vitro, and targeted deletion of AQP3 in mice results in considerably delayed wound healing [8,9]. Reduced migratory activity has also been reported in vascular endothelial cells from AQP1 null mice. In addition, AQP1 and/or AQP5 have been shown to increase tumor cell growth [10–12].

AQP5 exists on apical cellular membranes at immunologically active tissues such as submucosal glands and the alveolar epithelium in the lungs. Therefore, in the present study, we examined whether AQP5 affects inflammatory responses in terms of chemokine expression. We propose a new function of AQP5, which enhances chemokine expression through activation of ERK.

2. Materials and methods

2.1. Reagents

TNF-α and MG132 were obtained from Sigma (St. Louis, MO). A rabbit anti-AQP5 antibody was from Millipore (Temecula, CA), and rabbit anti-phospho-p44/p42 mitogen-activated protein kinase (MAPK) (ERK1/2) (Thr202/Thr204), anti-p44/p42 MAPK (ERK1/2) and anti-IκB-α antibodies were from Cell Signaling Technology (Danvers, MA). PD98059, SB203580, U0126, actinomycin D, and tetraethylammonium chloride were from Wako (Saitama, Japan).

2.2. Cell culture and treatments

MLE-12 (immortalized murine lung epithelial) cells and NIH-3T3 (immortalized murine fibroblast) cells were obtained from the American type culture collection. The cells were cultured as described previously [13]. Cells were serum-starved for 6 h before

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treatment with TNF-α. The cells were then treated with the vehicle or TNF-α in DMEM (serum-free). For inhibitor experiments, the cells were pretreated with 20 μM SB203580 (p38 inhibitor), 20 μM PD98059 (MEK1/2 inhibitor), or 5 μM MG132 for 1 h before TNF-α treatment.

2.3. ELISA for keratinocyte chemoattractant (KC)

Conditioned medium was centrifuged at 150*g* for 4 min and then stored at −80 °C until use. KC in the conditioned medium was assayed by a commercially available ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

2.4. Western blot analysis

Cells were washed with ice-cold PBS (pH 7.4) and then exposed to lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% (v/v) NP-40, 0.1% (w/v) SDS, 0.5% (w/v) deoxycholate, and 1% (v/v) protease inhibitor cocktail) for 30 min at 4 °C. Protein concentrations in lysates were measured using the bicinchoninic acid method with bovine serum albumin as the standard. Ten micrograms of proteins were separated by 10% SDS–polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane. The membrane was blocked with Blocking One-P, Blocking One, or 5% skim milk powder in PBS containing 0.1% Tween-20 (TBS) for 1 h at room temperature. After blocking, the membrane was incubated with affinity-purified rabbit anti-phospho-p44/p42 MAPK (1:1000), anti-p44/p42 MAPK (1:1000), or anti-AQP5 (1:1000) antibodies at 4 °C for 8 h. The membrane was then washed with TBS and incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG for 1 h at room temperature. Immunocomplexes were detected using ECL chemiluminescent reagents (Amersham, Waukesha, WI).

2.5. Real-time quantitative RT-PCR

After treatment with TNF-α for 1 h, the cells were collected in 1 ml of RNAiso Plus® reagent (Takara, Shiga, Japan) or ISOGEN (Nippon gene, Tokyo, Japan), homogenized on ice, and then total RNA was extracted according to the manufacturer's instructions. Reverse transcription was performed with PrimeScript® RT Master Mix (Takara). Real-time PCR was performed with SYBR® Premix Ex Taq™ (Takara) on a Chromo 4™ real-time PCR analysis system (Bio-Rad, Tokyo, Japan). Samples were run in duplicate. The thermal cycling program consisted of 95 °C for 3 min for polymerase activation, and then 40 cycles of denaturation (95 °C for 15 s) and annealing and extension (60 °C for 1 min). Reactions were quantified by selecting the amplification cycle when the PCR product of interest was first detected [threshold cycle (Ct)]. Data were analyzed by the comparative Ct method. The primer sequences used in this study were as follows: KC, forward 5′-TCGTCTTTCATATTGTATGGTCAAC-3′ and reverse 5′-CGAGACGAGACCAGGAGAAAC-3′; GAPDH, forward 5′-ACCATCTTCCAGGAGCGAGC-3′ and reverse 5′-CAGTCTTCTGGGTGGCAGTG-3′.

2.6. RNA interference

To knockdown mouse AQP5, siGENOME SMART pool® siRNA reagents for AQP5 were purchased from Dharmacon (Lafayette, CO). siRNA transfections were performed with DharmaFECT 1 Transfection Reagent (Dharmacon). Cells cultured in 12-well plates to 30–50% confluence were incubated with the siRNA transfection reagent mixture. At 42 h after transfection, the cells were

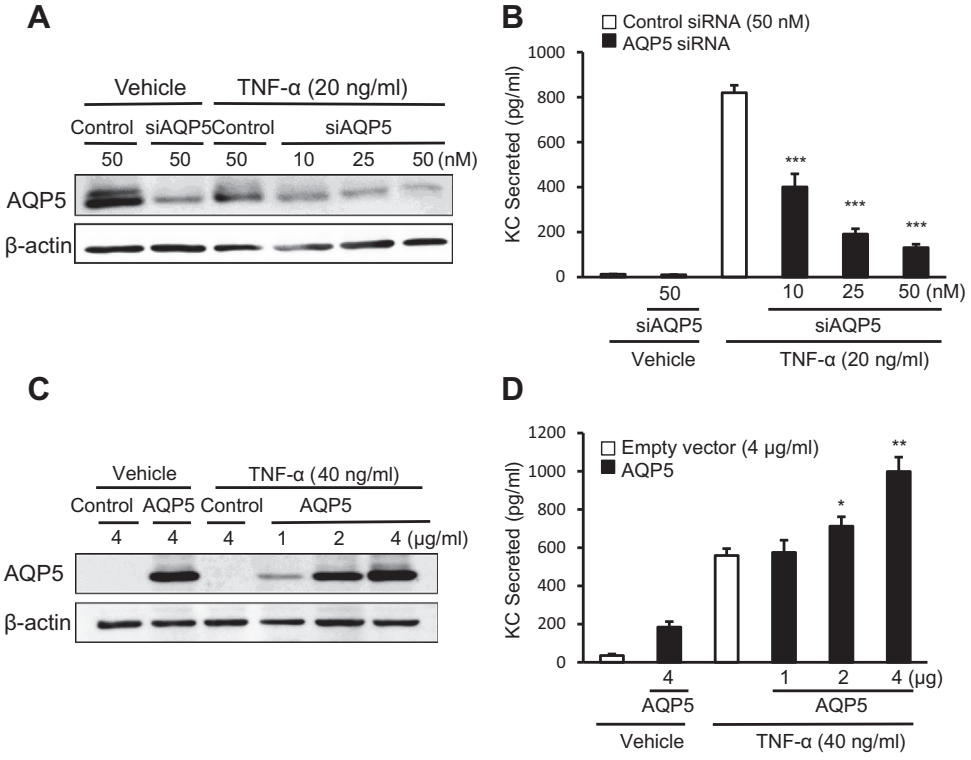

Fig. 1. Effect of AQP5 on TNF-α-induced KC secretion. MLE-12 cells were transfected with the indicated amount of mouse AQP5 siRNA for 48 h, and then treated with TNF-α (20 ng/ml) for 6 h (A and B). NIH-3T3 cells were transfected with the indicated amount of mouse AQP5 cDNA for 24 h, and then treated with TNF-α (40 ng/ml) for 6 h (C and D). Cellular proteins were analyzed by Western blotting (A and C), and secreted KC in the medium was analyzed by ELISA (B and D). Data represent the means ± S.E. from three independent experiments performed in duplicate. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with TNF-α-treated control cells.

serum-starved for 6 h and then treated with TNF-α for 1 or 6 h, followed by harvesting for analysis.

2.7. Plasmid constructs, transient transfection and luciferase assay

Mouse cDNA from MLE-12 cell was amplified by polymerase-chain reaction (PCR) using primers for AQP5, and then inserted into *Kpn* I and *Xba* I site of pcDNA 3.1(+). The NF-κB-luc construct was purchased from Stratagene (La Jolla, CA, USA). Transient transfection of plasmids was performed with HilyMax reagent (Dojindo, Kumamoto, Japan) according to the manufacturer's recommendations. Briefly, cells cultured in 12-well plates to 70–80% confluence were incubated with the DNA transfection reagent mixture. At 18 h after transfection, the cells were serum-starved for 6 h and then treated with TNF-α for 8 h. Luciferase activity was then measured using a luminometer (Lumat LB9507, EG&G Berthold) with a Dual luciferase assay kit (Promega). Co-transfection with pGL4.74 [hRluc/TK] (Promega), which carries Renilla luciferase, was performed to normalize the transfection efficiency.

2.8. Statistics

Statistical analyses were performed with one-way or repeated-measures analysis of variance (ANOVA) or the Student's two-tailed *t*-test for paired samples. A value of $p < 0.05$ was considered to be significant.

3. Results

MLE-12 cells show high expression of AQP5 protein. To investigate whether AQP5 affects inflammatory responses, the cells were transfected with specific siRNA against AQP5, and then KC secretion induced by TNF-α was measured by ELISA. As a result, endogenous AQP5 expression was decreased in a dose-dependent manner (Fig. 1A). KC levels in TNF-α (20 ng/ml)-containing medium were significantly higher than those in control medium (Fig. 1B). Interestingly, AQP5 siRNA transfection resulted in considerable attenuation of TNF-α-induced KC secretion with the most pronounced attenuation at 50 nM (80% of control siRNA-transfected cells). On the other hand, in NIH-3T3 cells, which did not express AQP5, transfection of AQP5 cDNA increased both AQP5 protein and TNF-α-induced KC secretion in a dose-dependent manner (Fig. 1C and D).

KC mRNA levels were determined by real-time PCR. TNF-α induced a 110-fold increase of KC mRNA levels in MLE-12 cells transfected with control siRNA. In good agreement with the data of KC protein secretion, AQP5 knockdown by siRNA attenuated the TNF-α-induced increase of KC mRNA in a dose-dependent manner (Fig. 2A). Conversely, transfection of AQP5 cDNA into NIH-3T3 cells resulted in a dose-dependent increase of TNF-α-induced KC mRNA (Fig. 2B).

We also examined the effect of AQP5 knockdown on KC mRNA expression over time and the dose–response of TNF-α in MLE-12 cells. In control cells, TNF-α-induced KC expression was biphasic,

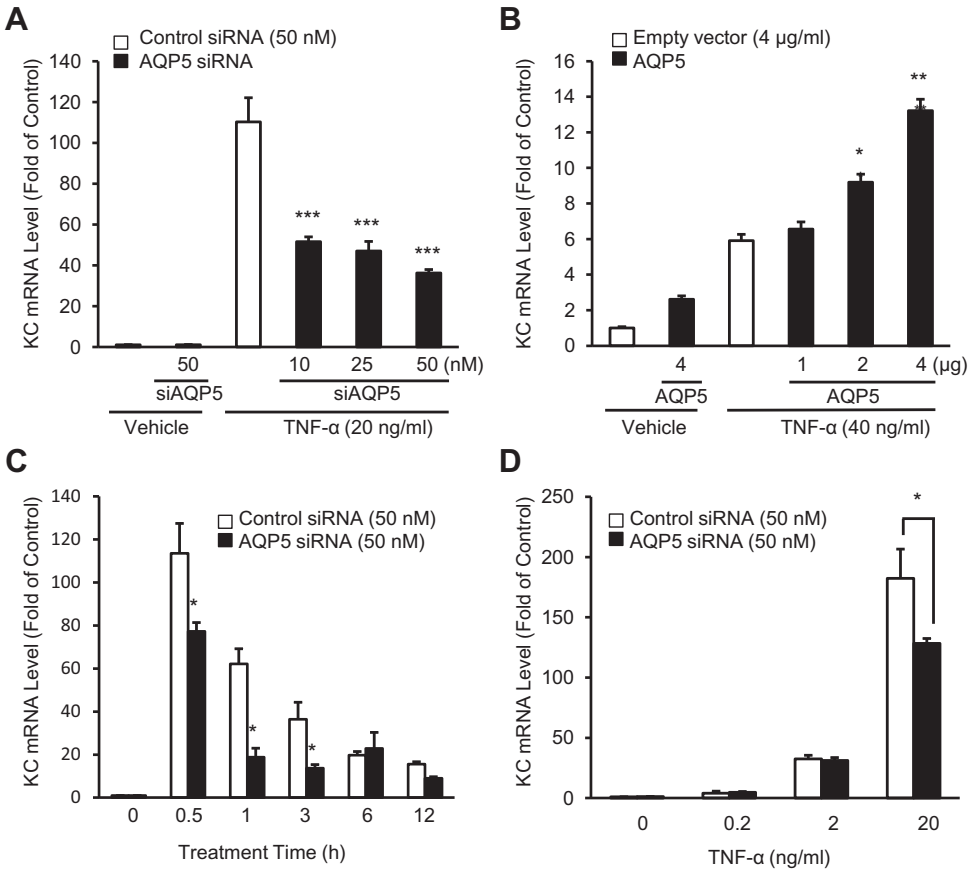

Fig. 2. Effect of AQP5 on TNF-α-induced KC mRNA expression in MLE-12 and NIH-3T3 cells. MLE-12 cells were transfected with the indicated amount of mouse AQP5 siRNA for 48 h and then treated with 20 ng/ml TNF-α for 1 h (A). NIH-3T3 cells were transfected with the indicated amount of mouse AQP5 cDNA (B). To analyze dose- and time-dependent effects of TNF-α, MLE-12 cells were transfected with 50 nM AQP5 siRNA, and then treated with the indicated concentrations of TNF-α for 1 h (C) or 20 ng/ml TNF-α for the indicated period (D). Total RNA was isolated and real-time quantitative RT-PCR was performed to determine the level of KC mRNA. Data represent the mean ± SE ($n = 3$). $^{*}p < 0.05$ and $^{***}p < 0.001$ compared with TNF-α-treated control cells.

an early and transient phase (1–3 h) and a prolonged and weak phase (6–12 h). AQP5 siRNA attenuated the early phase of KC mRNA expression, whereas the prolonged phase was unaffected (Fig. 2C). On the other hand, AQP5 siRNA attenuated KC mRNA expression in cells treated with 20 ng/ml TNF-α (Fig. 2D), which produced the maximal response, suggesting that AQP5 has a greater effect at high doses of TNF-α during the early phase.

TNF-α activates IκB kinases, resulting in translocation of NF-κB from the cytoplasm into the nucleus to activate transcription of target genes [14]. NF-κB plays an important role in the expression of KC [15]. We next examined the involvement of NF-κB in the increase of KC mRNA expression induced by AQP5. MG132, an inhibitor of NF-κB, significantly blocked TNF-α-induced KC mRNA expression in both control and AQP5 cDNA-transfected cells. The increase of KC expression induced by AQP5 was not significant in the presence of MG-132 (Fig. 3A). Luciferase assays were also performed with an artificial promoter plasmid containing three NF-κB sites. Induction of luciferase activity by TNF-α in AQP5 cDNA-transfected cells was considerably greater than that in control cells (Fig. 3B). In addition, translocation of the NF-κB component p65 into the nucleus was determined by Western blotting. TNF-α treatment resulted in a clear increase of nuclear p65 without changing the level of LAMNB1, a histone protein. In AQP5 cDNA-transfected cells, the level of nuclear p65 was significantly greater than that in control cells (Fig. 3C). Nuclear translocation of NF-κB requires dissociation of IκB, which is controlled by phosphorylation of IκB and the subsequent degradation induced by various extracellular signals, including TNF-α. As shown in Fig. 3D, TNF-α induced IκB degradation in both control and AQP5 cDNA-transfected cells. The decrease in of IκB in AQP5 cDNA-transfected cells was faster than that in control cells (Fig. 3D), suggesting that AQP5 potentiated the TNF-α-induced activation of NF-κB.

To exclude the possibility that AQP5 transfection affected the stability of KC mRNA, the levels of KC mRNA were monitored for

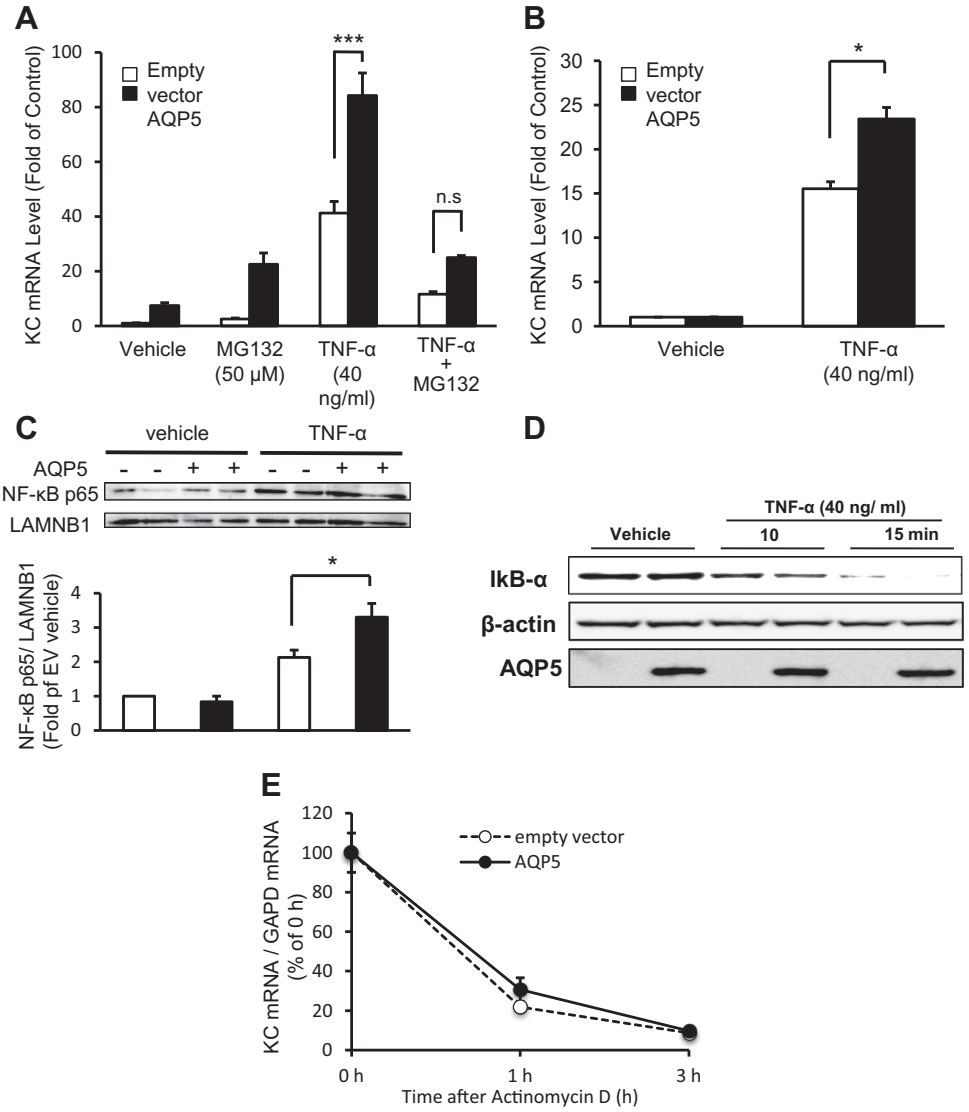

Fig. 3. Effect of AQP5 on NF-κB activity and KC mRNA stability. NIH-3T3 cells were transfected with AQP5 cDNA, and then pretreated with or without 5 μM MG132 for 1 h. The cells were then treated with 40 ng/ml TNF-α for 1 h. Total RNA was isolated and real-time quantitative RT-PCR was performed to determine the level of KC mRNA (A). Cells were transfected with AQP5 and a NF-κB promoter construct, and then treated with 40 ng/ml TNF-α. At 10 h after treatment, luciferase activity was measured (B). Cells were treated with 40 ng/ml TNF-α for 1 h, and then the amount of p65 in the nuclear protein fraction was determined by Western blotting (C). Cells were treated with 40 ng/ml TNF-α for 0, 10, and 15 min, after which cell lysates were prepared and the levels of IκBα, β-actin and AQP5 were determined by Western blotting (D). Cells were treated with 5 μg/ml actinomycin D and 40 ng/ml TNF-α for 0, 1, and 3 h. Total RNA was isolated and real-time quantitative RT-PCR was performed (E). Data represent the mean ± SE ($n = 3$). $^{*}p < 0.05$ and $^{***}p < 0.001$ compared with TNF-α-treated control cells.

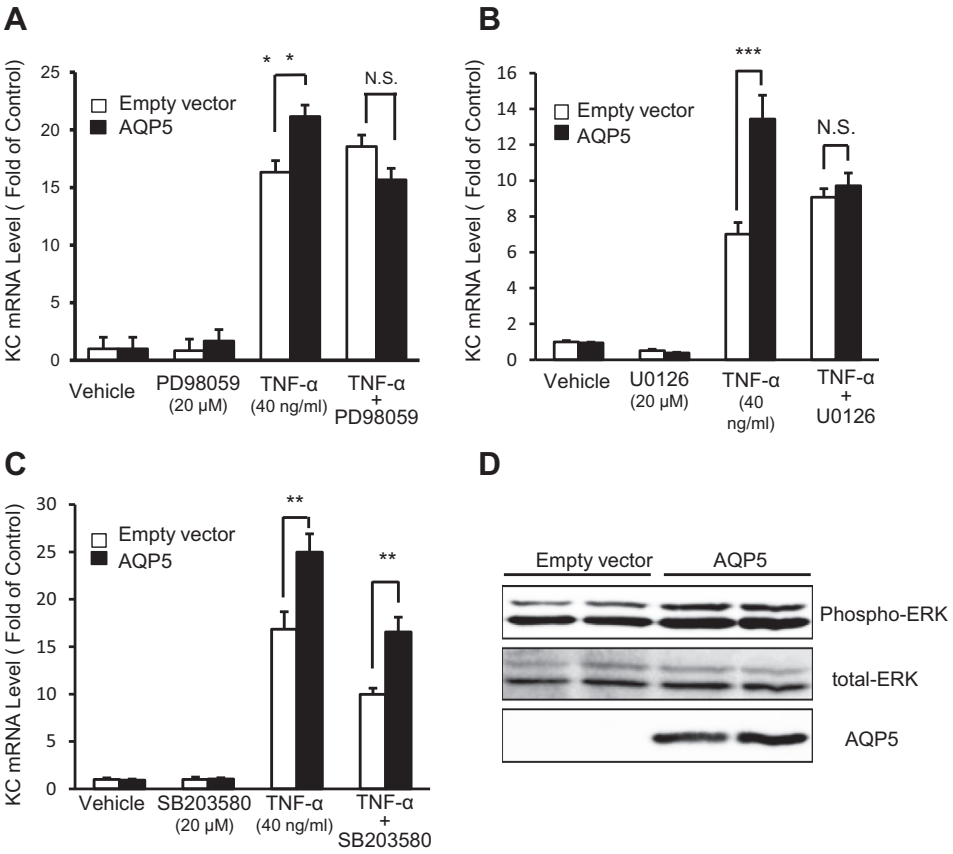

Fig. 4. Involvement of ERK in the AQP5-mediated increase of KC expression. NIH-3T3 cells were transfected with AQP5 cDNA for 24 h, and then pretreated with or without 20 μM PD98059 (A), U0126 (B), or SB2003580 (C) for 1 h, followed by treatment with 40 ng/ml TNF-α for 1 h. Total RNA was isolated and real-time quantitative RT-PCR was performed. NIH-3T3 cells were transfected with AQP5 cDNA for 24 h. Cell lysates were prepared and the levels of phosphorylated and total ERK were determined by Western blotting with anti-phospho-p44/p42 MAPK and anti-p44/p42 MAPK antibodies (D). Data represent the mean ± SE ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with TNF-α-treated control cells.

3 h in the presence of actinomycin D. A significant change in the stability of KC mRNA was not observed in AQP5 cDNA-transfected cells compared with that in control cells (Fig. 3E).

TNF-α activates not only NF-κB, but also MAPKs as downstream signaling of its receptors [16,17]. To investigate whether MAPKs were involved in the potentiated response to TNF-α in AQP5-expressing cells, we examined the effects of MAPK inhibitors. Cells were pre-treated with PD98059 (20 μM), U0126 (20 μM), or SB203580 (10 μM) for 1 h, and then TNF-α-induced KC mRNA expression was determined. Both MEK-ERK inhibitors, PD98059 and U0126, partly blocked KC mRNA expression in AQP5 cDNA-transfected cells, whereas they did not change the response in control cells (Fig. 4A and B). In the presence of these inhibitors, the increased KC mRNA expression was abolished in AQP5 cDNA-transfected cells, suggesting ERK may be involved in the increased response of AQP5 cDNA-transfected cells. On the other hand, SB203580, a p38 inhibitor, blocked the response of both control and AQP5 cDNA-transfected cells. However, the level of KC mRNA in AQP5 cDNA-transfected cells was significantly higher than that in control cells (Fig. 4C). Finally, the steady state level of phosphorylated ERK was determined by Western blotting. Interestingly, the steady state level of phosphorylated ERK was considerably increased in AQP5 cDNA-transfected cells compared with that in control cells, suggesting that AQP5 expression induced ERK activation.

4. Discussion

KC is one of the most important mediators secreted from airway epithelial cells, which activates neutrophil influx into lung tissue [18,19]. In the present study, we examined the effect of AQP5 on KC secretion and mRNA expression induced by TNF-α. All data presented here indicate that AQP5 increases KC expression in response to TNF-α. In MLE-12 cells, which highly express AQP5, knockdown of endogenous AQP5 resulted in a considerable decrease of both KC mRNA and protein expression induced by TNF-α (Figs. 1B and 2A). In contrast, transfection of AQP5 cDNA into NIH-3T3 cells, which has no endogenous AQP5 expression, caused an increase of both KC mRNA and protein expression (Figs. 1D and 2B). The overexpression of AQP5 also increased membrane water permeability in NIH3T3 cells assessed by stopped-flow light scattering assay (Supplementary Fig. 1), suggesting that AQP5 is localized on plasma membrane, as well as endogenous AQP5. The potentiation of KC expression was completely dependent on the level of AQP5 expression. Surprisingly, knockdown of AQP5 blocked about 80% of KC secretion in MLE-12 cells (Fig. 1B). In addition, AQP5 increased KC expression not only induced by TNF-α, but also IL-1β (Supplementary Fig. 2), suggesting that the increased response induced by AQP5 is not dependent on the TNF receptor.

In good agreement with the increase of KC mRNA and protein expression, AQP5 increased NF-κB activation induced by TNF-α, as determined by luciferase assays with artificial promoter-containing NF-κB sites (Fig. 3B). Therefore, we assume that AQP5 increases KC expression through increased activation of the transcription factor NF-κB. To support this idea, the increase of KC expression induced by AQP5 was considerably attenuated by MG132, an NF-κB inhibitor (Fig. 3A). The amount of p65 in the nuclear fraction of TNF-α-treated cells and IκB degradation were potentiated in AQP5-expressing cells (Fig. 3C and D). Thus, AQP5

may increase the expression of not only KC, but also other NF-κB-dependent inflammatory genes such as inducible nitric oxide synthase, cyclooxygenase-2, IL-1β, and IL−6. In support of this idea, transfection of AQP5 siRNA significantly attenuated the TNF-α-induced IL-6 and TNF-α mRNA expression in MLE-12 cells (Supplementary Fig. 3), although we did not examine other genes.

The increased response to TNF-α induced by AQP5 is thought to be caused by activation of ERK. In the present study, both ERK inhibitors, PB98059 and U0126, blocked the increase of KC mRNA expression induced by AQP5, although they did not change the expression level of KC mRNA in control cells (Fig. 4A and B). These data clearly suggest that ERK-dependent signals are involved in the increased KC expression in AQP5-expressing cells. In addition, the steady state level of ERK phosphorylation in AQP5-expressing cells was considerably higher than that in control cells (Fig. 4D). This result is consistent with that of Kang et al. [20]. In their report, overexpression of AQP5 induced cell proliferation and ERK phosphorylation in colon cancer cells [20]. ERK is associated with KC secretion as well as cell proliferation. For example, Farmand et al. reported that treatment of mouse lung epithelial cells with a Fas-activating monoclonal antibody resulted in KC secretion that was blocked by ERK inhibitors [21]. On the other hand, p38 inhibitor, did not affect KC expression. We have previously reported that LPS changes AQP5 subcellular localization from plasma membrane to intracellular fraction in MLE-12 cells through p38-dependent pathway [22]. In the present study, however, treatment of TNF-α for 1 h did not change membrane water permeability in MLE-12 and NIH-3T3 cells (data not shown). Therefore, we might be able to exclude the possibility that change in subcellular localization of AQP5 is related to the AQP5-induced ERK phosphorylation and potentiated KC expression.

The airway epithelium is positioned at the interface with the environment, and thus plays a key role in the host defense system [23]. Airway epithelial cells respond to pathogen exposure by increasing the production of chemokines and cytokines. The activities of the innate immune system are closely linked to inflammatory processes. All major diseases of the lung involve mechanisms of the innate or adaptive immune system. Asthma and chronic obstructive lung disease are chronic inflammatory diseases in which cytokines and other mediators secreted from the airway epithelium are likely to play a critical role. In the present study, we propose a new function of AQP5 as an inflammatory response enhancer. This function may contribute to the innate immune system by increasing cytokine production. On the other hand, Towne et al. and other investigators have shown that inflammatory stimuli such as TNF-α decrease the expression of AQP5 [24]. This decrease of AQP5 might have a role in feedback inhibition of inflammatory responses to prevent prolonged activation of the inflammatory system. However, further studies are needed to test this hypothesis.

In summary, the present study clearly indicates that AQP5 regulates proinflammatory cytokine-induced gene expression in lung cells. This new function is likely to be at least partly because of the increased activation of ERK and NF-κB induced by AQP5. It is uncertain whether this new function is related to transport of water or other small molecules through AQP5, and whether other AQPs have a similar effect. However, further characterization of this function of AQPs may provide a new insight into the treatment of inflammatory diseases.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.047.

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