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CELLULAR & MOLECULAR BIOLOGY LETTERS
http://www.cmbl.org.pl

Received: 11 July 2011
Final form accepted: 02 November 2011
Published online: 09 November 2011

Volume 17 (2012) pp 1-10
DOI: 10.2478/s11658-011-0031-y

Short communication

p38 MITOGEN-ACTIVATED PROTEIN KINASE MODULATES EXOCRINE SECRETION IN RABBIT LACRIMAL GLAND

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Abstract: The lacrimal gland (LG) is an exocrine gland important for secretion of the tear film. The kinase p38 has important signal transduction functions, e.g. in gene transcription, but has previously not been known to modulate exocrine secretion. The aim of the current study was to investigate the role of p38 in carbachol (Cch)-induced LG secretion in LG acinar cells *in vitro*. Western blotting was used to determine the phosphorylation status of p38 and p42/44 and determine expression of p38 isoforms. To determine the effect of p38 inhibition on LG secretion, PD 169316, a general p38 inhibitor, and SB 239063, an inhibitor of p38α and β, were added to the cells prior to secretion measurements. The results revealed activation of p38 mediated by Cch stimulation and inhibition of Cch-induced secretion as a result of p38 inhibition. The inhibition was observed with PD 169316 isoforms, but not with SB 239063. The p38δ isoform was shown to have robust expression both by Western blotting of acinar cells and immunofluorescence of the whole gland. In conclusion, p38 activation mediates secretion in cholinergic stimulation of rabbit LG cells.

Key words: p38 mitogen-activated protein kinase, Lacrimal gland, Exocrine glands, Signal transduction, Cholinergic receptors

INTRODUCTION

Mitogen-activated protein kinases (MAPKs) are a group of serine/threonine protein kinases which have been shown to be important in signal transduction of numerous cell types. One subfamily of these kinases is the p38 family.

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Abbreviations used: Cch – carbachol; JNK – c-jun N-terminal kinase; DAG – diacylglycerol; IP_3 –inositol triphosphate; LG – lacrimal gland; MAPK – mitogen-activated protein kinase; PBS – phosphate buffered saline

It consists of four isoforms: α, β, γ and δ. Activation of p38 occurs through phosphorylation of threonine 180 and tyrosine 182 [1]. The downstream kinase activity of p38 can regulate gene expression, protein turnover and endocytosis of membrane receptors [2].

The LG is an exocrine gland producing the aqueous layer of the tear film. The tear film is vital for the ocular surface to e.g. prevent pathogenic infection, provide oxygen to the corneal cells and moisten the eye. The secretion is regulated mainly by the autonomic nervous system, with acetylcholine being an important inducer of secretion [3]. The acetylcholine signaling pathway in the LG has previously been investigated in several studies, revealing the muscarinic receptor 3 to be the receptor involved, with subsequent activation of phospholipase C leading to production of inositol triphosphate (IP_3) and diacylglycerol (DAG) [4]. IP_3 mediates a rise in intracellular calcium concentrations while DAG activates protein kinase C. Studies in rat LG have shown that cholinergic stimulation mediates phosphorylation of p42/44 that is inhibitory to secretion [5].

The current study reports a new effect of p38 activation. Stimulation of LG cells with a cholinergic agonist activates p38, and inhibition of p38 leads to diminished secretion, implicating a novel role of p38 in modulating exocrine secretion.

MATERIALS AND METHODS

Animals

New Zealand White rabbits were used in concordance with the Guidelines of the Ethical Committee for Animal Experiments (Linköping, Sweden) and the ARVO Statement for the use of animals in ophthalmic and vision research. LGs were isolated from female rabbits weighing approximately 1.7-2.0 kg (ESF products Estuna AB, Norrtälje, Sweden). The animals were sedated with an intramuscular injection of 0.25 ml mixture of Zolazepam (50 mg/ml) (Virbac, Fort Worth, TX) and Medetomidin (5 mg/ml) (Orion, Espoo, Finland) and sacrificed with 2 ml of sodium pentobarbital (60 mg/ml) (Apoteket AB, Stockholm, Sweden) injected in the marginal ear vein. The LGs were removed without removal of the eye bulb.

Antibodies

The antibodies used in Western blotting were diluted in 5% bovine serum albumin in phosphate-buffered saline (PBS) with 0.2% Tween, and for immunofluorescence in 5% goat serum in PBS. The following antibodies and dilutions were used for Western blotting: phosphorylated p38, dilution of 1:10,000, No. 4511, Cell Signaling Technology, Danvers, MA; p38α,β,γ, 1:5000, No. 9212, Cell Signaling Technology; p38δ, 1:2000, No. 65029, ProteinTech Group, Manchester, UK; p38γ, 1:1000, No. MAB1347, R & D Systems, Minneapolis, MN; total p42/44 MAPK 1:10,000, No. 4696, Cell Signaling Technology; P-p42/44 MAPK, 1:4000, No. sc-7383 Santa Cruz

Biotechnology, Santa Cruz, CA; beta-actin 1:10,000, No. ab8229, Abcam, Cambridge, MA; horseradish peroxidase-conjugated goat anti-rabbit, 1:15,000, No. ab6721-1, Abcam; goat anti-mouse, 1:15,000, No. 31444, Pierce, Rockford, IL. For immuno-fluorescence, the p38δ antibody from above was used at dilution 1:1000, and the p38γ antibody at dilution 1:100. For fluorescence detection, goat anti-mouse antibody conjugated with Alexa 488 (1:200) and phalloidin conjugated with Alexa 546 (1:100), both from Molecular Probes, Invitrogen Corp. Carlsbad, CA, were used.

LG single cell isolation and stimulation

LG single acinar cells were isolated and cultured as previously described [6]. Cells were seeded in 48-well plates for secretion assay (6.4×10^5 cells/well) and 6-well plates for lysate preparation in Western blotting experiments (6.4×10^6 cells/well). The cells were cultured for 2-3 days in order for them to reorganize into acini-like structures [6] and then washed twice in Hank's culture medium complemented with 10 mM HEPES and 1 mM calcium chloride (all from Sigma-Aldrich, St Louis, MO).

***In vitro* secretion assay**

Cells used in secretion experiments were preincubated with or without p38 antagonists PD 169316 (2 μM) or SB239063 (2 μM) for 30 min, and thereafter supplemented with carbachol (Cch) at a final concentration of 100 μM (all from Sigma-Aldrich) and incubated for an additional 5, 15 or 30 min as indicated. The IC_{50} of PD 169316 and SB 239063 is 89 and 44 nM, respectively, and the concentrations utilized are well in concordance with concentrations reported as effective in other tissues [7-8]. Supernatants were then centrifuged at 13,000 x *g* for 5 min and the aspirated supernatants were stored at -20°C. Secretion was measured using a marker for secretion, β-hexosaminidase [6]; the methodology is described elsewhere [9]. In brief, a substrate for β-hexosaminidase was added to the supernatant, yielding a fluorescent product which was measured in a Flurolog 3-22 or Fluoromax 3 fluorometer (Horiba Jobin Yvon, Edison, NJ) with excitation at 365 nm and emission at 460 nm. The Cch response was set to 100%.

Western blotting

Cells grown in 6-well plates were stimulated with carbachol for 5 or 30 min. The cells were lysed in a lysis buffer containing 100 mM Tris-HCl pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% sodium deoxycholate, 2 mM Na_3VO_4 and 100 mM NaF with one Protease Inhibitor Cocktail Mini Tablet (Roche Diagnostics GmbH, Mannheim, Germany) per 10 ml of extraction buffer. Lysates were centrifuged at 11,000 x *g* for 20 min at 4°C, and the supernatants were used for Western blotting. Protein concentrations of the homogenates were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Separation, electroblotting and Western blotting of 10-15 μg of total protein were performed. The membranes were blocked with 5% dry milk in PBS with 0.2% Tween-20. Incubation with primary antibody was performed

over night at 4°C, and secondary antibody for 1-2 hours at room temperature. Membranes were stripped and incubated with β-actin primary antibody. The intensity of the phosphorylated protein signal was normalized to the respective β-actin signal.

Immunofluorescence

Immunofluorescence methodology used was described elsewhere [10]. In brief, isolated LGs were fixed and sectioned into 5 μm sections. The sections were thawed, encircled with a PAP pen and permeabilized for 5-10 min with 0.2% Triton X-100 in PBS. Blocking of the slides was performed with 5% goat serum and the sections were thereafter incubated with primary mouse anti-p38δ or p38γ antibody overnight at 4°C. Incubation with secondary goat anti-mouse antibody, conjugated with Alexa 488, and phalloidin, conjugated to Alexa 546, was performed and sections were evaluated using a Nikon Eclipse E600 Microscope with a Nikon C1 Confocal system (Nikon, Japan). Images were compiled in GIMP 2.6 (http://www.gimp.org). As negative controls for immunofluorescence, the primary antibody was omitted.

Statistical analysis

Throughout the study, results are presented as mean ± standard error of the mean (SEM). For Western blotting, three or four individual experiments were analyzed and statistical assessments were made with the Mann-Whitney test where Gaussian distribution is not assumed. For secretion measurements, one-way ANOVA with Tukey's multiple comparisons post test was used and every n represents LG cell preparation from two animals, with three replicates performed for each n. $p < 0.05$ was considered statistically significant.

RESULTS

Effects of cholinergic stimulation on phosphorylation of p38 and p42/44

Measurements of p38 activation in cells stimulated with Cch were performed by Western blotting with specific antibodies for p38 in phosphorylated form as well as total p38. The results are shown in Fig. 1A, and show significantly higher immunoreactivity for phosphorylated p38 in LG cells stimulated with Cch for 30 min compared to unstimulated cells (1.35 ± 0.20 and 0.53 ± 0.09 respectively, $n = 3\text{-}4$, $p < 0.05$). The phosphorylation of p38 was observed also after 5 min of Cch stimulation. Phosphorylation of p42/44 was also investigated and the results are shown in Fig. 1C-D. No statistically significant changes in p42/44 phosphorylation were seen after 5 or 30 min of Cch stimulation.

Effects of p38 inhibitors on LG secretion

To investigate if the phosphorylation of p38 affected secretion from the cells, they were preincubated with p38 inhibitors for 30 min before addition of Cch. After 5 min of Cch stimulation, there was no significant difference between the Cch stimulated cells with or without PD 169316, an inhibitor of all p38 isoforms

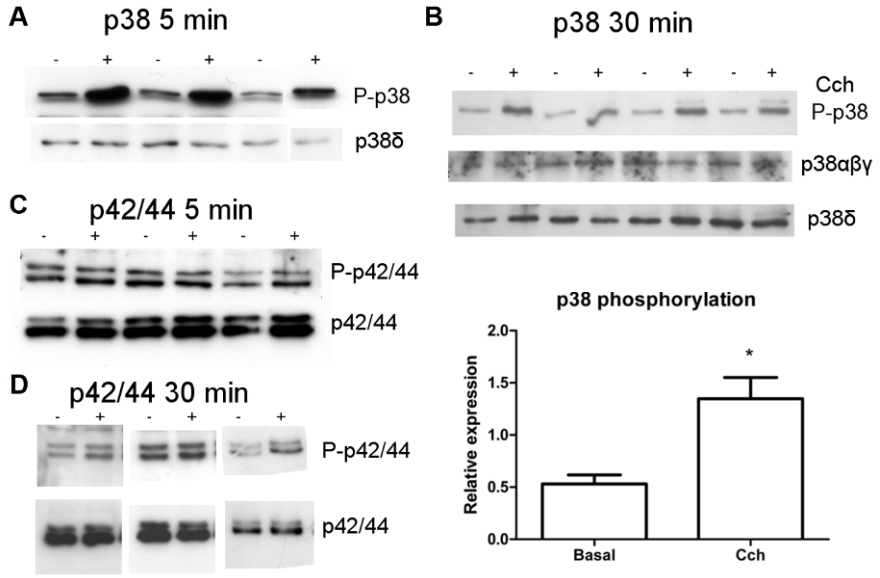

Fig. 1. Expression and phosphorylation of p38 and p42/44. LG cells stimulated for 5 or 30 min with (+) or without (-) 100 μM Cch were lysed and p38 (A,B) and p42/44 (C,D) protein presence was assessed by Western blotting. Phosphorylated p38 (P-p38) band intensity at 30 min was measured and normalized to actin of the stripped membrane. N = 3-4, *p < 0.05. B includes densitometric measurement of phosphorylated protein in Cch-stimulated cells compared to basal.

(Fig. 2A). PD 169316 significantly decreased carbachol-induced secretion after 15 min (from 100% to 50.6 ± 11.5%, $p < 0.01$) and 30 min (from 100 % to 67.9 ± 6.95%, $p < 0.01$) of Cch stimulation, as seen in Fig. 2B-C.

Incubation with an isoform-specific inhibitor against p38αβ, SB 239063, resulted in no statistically significant changes (Fig. 3). This suggests that the γ or δ isoform, or possibly both, were responsible for the decreased secretion.

Cellular expression of p38δ and p38γ

The cellular expression of p38δ and p38γ was investigated by immunofluorescence. Cryo-sectioned LGs were separately stained for the p38 isoforms, and the results are seen in Fig. 4 and 5, where green demonstrated immunoreactivity for p38 isoform and red is filamentous actin. In Fig. 4A, clear staining of p38δ is seen in acinar cells, and to a lesser extent in ductal cells and cells in or surrounding the blood vessel. Fig. 4B shows an acinus where clear p38δ staining is seen in the cytosol but not in the nuclei. Fig. 5 shows immunoreactivity of p38γ. Immunohistochemistry of LG tissue showed no immunoreactivity to p38γ, but a band of expected molecular weight, approximately 38 kDa, was detected by Western blotting, as shown in Fig. 5B. Negative controls showed very low labeling compared to p38δ immunoreactivity, similar to that seen for p38γ (data not shown).

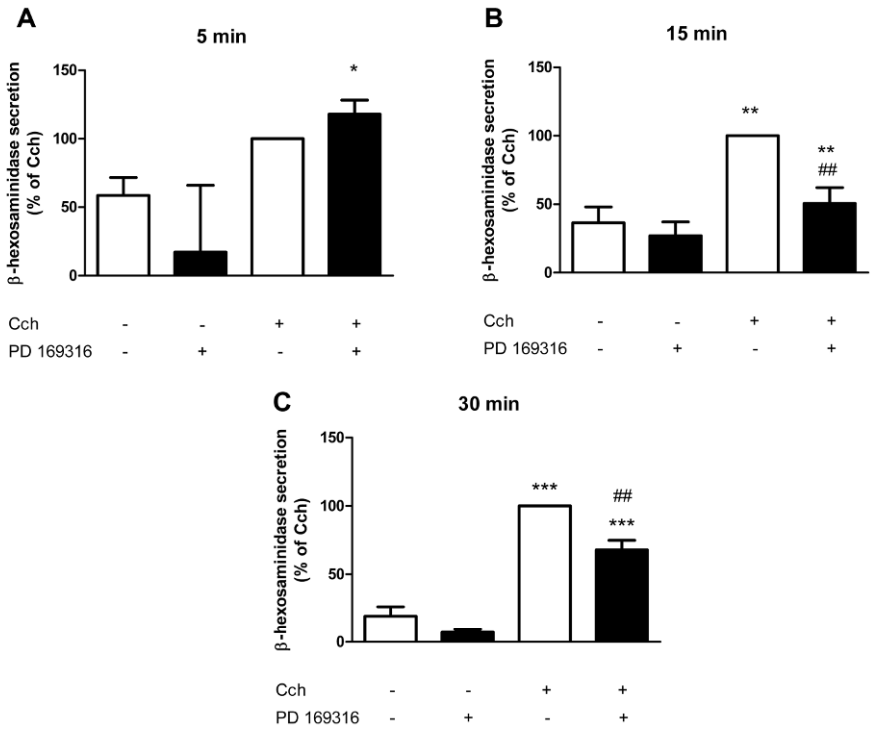

Fig. 2. Effect of p38 inhibition on LG secretion. Cultured rabbit LG acinar cells were preincubated for 30 min with the p38 inhibitor PD 169316 (2 μM) and thereafter incubated for 5 (A), 15 (B) or 30 min (C) with or without 100 μM Cch. Measurements of β-hexosaminidase activity in the supernatants of the cells were performed, and Cch-induced secretion was set to 100%. *,**, *** = significant difference from non-stimulated cells ($p > 0.05$, 0.01 and 0.001, respectively), ## = significant difference from Cch-stimulated cells ($p < 0.01$) n = 3-5.

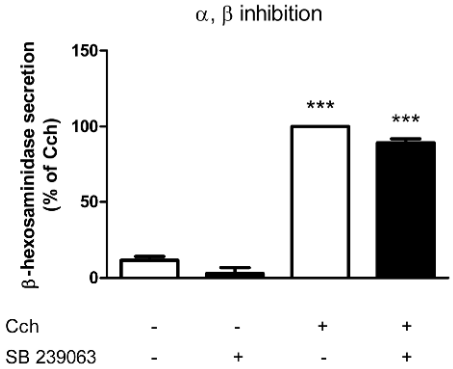

Fig. 3. Effect of p38αβ inhibition on LG secretion. LG cells were preincubated with 2 μM SB 239063 for 30 min prior to 30 min with or without 100 μM Cch. Secretion was assessed using β-hexosaminidase activity measurement, and the results are shown as % of the Cch-induced secretion.*** = significant difference from non-stimulated cells (***$p < 0.001$, n = 3).

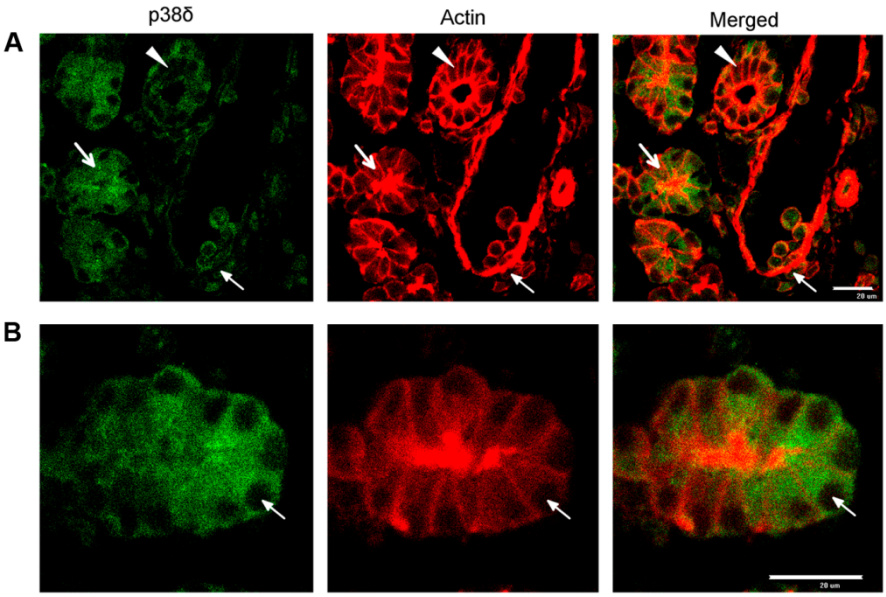

Fig. 4. Cellular expression of p38δ. Cryo-sectioned tissue was labeled with a specific p38δ antibody and visualized with a secondary anti-mouse antibody conjugated with Alexa-488. Filamentous actin is counterstained with phalloidin conjugated with Alexa-546. Green shows p38δ while red indicates filamentous actin. Bars are 20 μm. A – Staining is seen in the cytosol of acinar cells (line arrow), and at lower intensity in ductal cells (arrow head) and cells in and surrounding the vessel (filled arrow). B – p38δ expression in an acinus. The staining occurs primarily in the cytosol and not in the nuclei (indicated by an arrow).

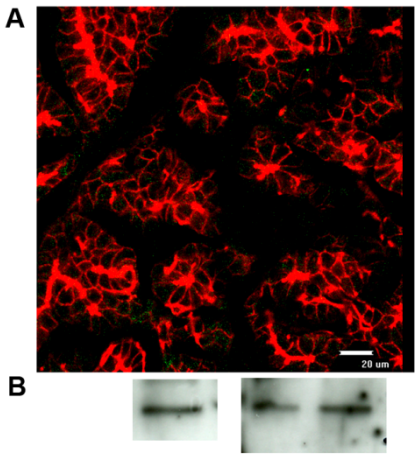

Fig. 5. Immunoreactivity of p38γ in rabbit LG. No immunoreactivity could be detected by immunohistochemistry (A), while a band of expected molecular weight was obtained by Western blotting (B). Bar is 20 μm and Western blotting was performed on three separate animals.

DISCUSSION

Previous studies have shown p38 to be involved in several important cell processes both in the nucleus and in the cytosol [2]. The activation of p38 in epithelial cells of exocrine tissues has been shown to modulate cell differentiation [11] and apoptosis [12], and mediate inflammatory responses [13]. To our knowledge, no previous studies of p38 function in the LG have been conducted other than for the use as a control for specificity of an inhibitor for another MAPK [14]. This study reveals a new function of the actions of p38 where it regulates exocrine secretion.

Cch stimulation mediated phosphorylation of p38, but not p42/44 (Fig. 1). Several studies have been performed investigating the role of MAPKs in lacrimal secretion, most of them regarding p42/44 [5,15-17], but also c-Jun N-terminal kinase (JNK) [14]. In rat LG, Cch-induced phosphorylation of p42/44 inhibits secretion [5,17]. In monkey LG, the Cch-induced secretion was found to be independent of p42/44 [16]. The results presented here showed no statistically significant p42/44 phosphorylation by Cch stimulation, even if phosphorylation was observed in some samples. Hence, the results in this study suggest that the role of p42/44 in rabbit LG is more similar to monkey than rat.

The results of this study showed that inhibition of p38αβ by SB 239063 gave no effect on secretion, while the inhibition of all p38 isoforms with PD 169316 diminished Cch-induced secretion. Consequently, the regulation of secretion was most likely exerted through p38γ, p38δ or both. Currently, no further isoform-specific inhibitors are accessible, and it is therefore problematic to make a more distinct determination of the isoform activated. The expression patterns of these p38 isoforms have not yet been investigated in LG, but in two other exocrine glands of humans, the salivary and mammary glands, the expression of p38γ is low while the expression of p38δ is high [18]. Therefore, p38δ is a strong candidate to have a role in exocrine tissues and to be responsible for the observed regulation of secretion. Pronounced expression of p38δ is also observed by Western blotting (Fig. 1) and immunofluorescence (Fig. 4). The expression of p38γ was investigated, and expression of the protein was indicated by Western blotting, although no immunohistochemical detection could be obtained. The effect of p38 inhibition was seen after 15 and 30 min, but not after 5 min (Fig. 2). The explanation for this may be that the sensitivity of the assay decreases with short incubation times, since the changes are smaller, and the inhibition is harder to detect.

However, there is no tendency of inhibition after 5 min, which contradics this hypothesis. It is also possible that p38 is involved in a certain part of the signal transduction pathway activated by Cch, which has a slower mechanism.

This study concludes that p38 is phosphorylated upon Cch stimulation, and that p38γδ inhibition decreases Cch-induced secretion, with p38δ as the probable actor.

Acknowledgements. The study was funded by the Linnaeus University Faculty and Crown Princess Margaretha Eye Research Foundation.

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