Cellular Signalling (2017.7) Volume 35:188-196.

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Activation of muscarinic receptors prevents TNF-α-mediated intestinal epithelial barrier disruption through p38 MAPK.

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Keywords: inflammatory bowel disease, tumor necrosis factor, intestinal barrier, muscarinic acetylcholine receptor, histamine receptor, p38 MAPK

Abbreviations: CCh, carbachol; EGFR, epidermal growth factor receptor; GPCR, G protein-coupled receptor; IBD, inflammatory bowel diseases; MAPK, mitogen-activated protein kinase; MT-7, muscarinic toxin 7; PKC, protein kinase C; PLC, phospholipase C; TACE, TNF-α converting enzyme; TAPI-0, TNF-alpha protease inhibitor-0; TER, transepithelial electrical resistance; TNF-α, tumor necrosis factor-α

ABSTRACT

Intestinal epithelial cells form a tight barrier to act as selective physical barriers, repelling hostile substances. Tumor necrosis factor-α (TNF-α) is a well characterized pro-inflammatory cytokine which can compromise intestinal barrier function and the suppression of TNF-α function is important for treatment of inflammatory bowel disease (IBD). In this study, we investigated the contribution of G-protein-coupled receptor (GPCR)-induced signalling pathways to the maintenance of epithelial barrier function. We first demonstrated the existence of functional muscarinic M3 and histamine H1 receptors in colonic epithelial cell HT-29/B6. As we previously reported, muscarinic M3 receptor prevented TNF-α-induced barrier disruption through acceleration of TNF receptor (TNFR) shedding which is carried out by TNF-α converting enzyme (TACE). M3 receptor-mediated suppression of TNF-α function depends on $G\alpha_{q/11}$ protein, however, histamine H1 receptor could not ameliorate TNF-α function, while which could induce $G\alpha_{q/11}$ dependent intracellular Ca^{2+} mobilization. We found that p38 MAPK was predominantly phosphorylated by M3 receptor through $G\alpha_{q/11}$ protein, whereas H1 receptor barely upregulated the phosphorylation. Inhibition of p38 MAPK abolished M3 receptor-mediated TNFR shedding and suppression of TNF-α-induced NF-κB signalling. The p38 MAPK was also involved in TACE- mediated EGFR transactivation followed by ERK1/2 phosphorylation. These results indicate that not H1 but M3 receptor-induced activation of p38 MAPK might contribute to the maintenance of epithelial barrier function through down-regulation of TNF-α signalling and activation of EGFR.

ABSTRACT

Intestinal epithelial cells form a tight barrier to act as selective physical barriers, repelling hostile substances. Tumor necrosis factor-α (TNF-α) is a well characterized pro-inflammatory cytokine which can compromise intestinal barrier function and the suppression of TNF-α function is important for treatment of inflammatory bowel disease (IBD). In this study, we investigated the contribution of G-protein-coupled receptor (GPCR)-induced signalling pathways to the maintenance of epithelial barrier function. We first demonstrated the existence of functional muscarinic M3 and histamine H1 receptors in colonic epithelial cell HT-29/B6. As we previously reported, muscarinic M3 receptor prevented TNF-α-induced barrier disruption through acceleration of TNF receptor (TNFR) shedding which is carried out by TNF-α converting enzyme (TACE). M3 receptor-mediated suppression of TNF-α function depends on $G\alpha_{q/11}$ protein, however, histamine H1 receptor could not ameliorate TNF-α function, while which could induce $G\alpha_{q/11}$ dependent intracellular Ca^{2+} mobilization. We found that p38 MAPK was predominantly phosphorylated by M3 receptor through $G\alpha_{q/11}$ protein, whereas H1 receptor barely upregulated the phosphorylation. Inhibition of p38 MAPK abolished M3 receptor-mediated TNFR shedding and suppression of TNF-α-induced NF-κB signalling. The p38 MAPK was also involved in TACE- mediated EGFR transactivation followed by ERK1/2 phosphorylation. These results indicate that not H1 but M3 receptor-induced activation of p38 MAPK might contribute to the maintenance of epithelial barrier function through down-regulation of TNF-α signalling and activation of EGFR.

1. Introduction

Intestinal epithelial cells form barrier to act as selective physical barriers, maintaining bacteria and maintain intestinal homeostasis. Disruption of barrier function of intestinal epithelium is one of the pathological features of inflammatory bowel disease (IBD) [1, 2].

Tumor necrosis factor-α (TNF-α) is a well characterized pro-inflammatory cytokine which could induce dysregulation of intestinal barrier function. TNF-α is secreted from monocytes and macrophages, which could bind to epithelial TNF receptors (TNFRs) and drive inflammatory signalling pathways. Activation of TNF-α signalling could induce epithelial barrier disruption in intestinal mucosal tissue and cultured epithelial monolayers [3, 4]. Expression level of TNF-α is upregulated in IBD patients, and suppression of TNF-α action is focused as the clinical target for IBD [5]. In fact, anti-TNF agents were developed and applied for the treatment of IBD (e.g. infliximab) [6]. Nuclear factor κB (NF-κB) is a main signalling mediator of TNF-α. NF-κB upregulates expression of various inflammation-related genes including inflammatory factors, cytoskeletal proteins and apoptotic factors to accelerate dysregulation of intestinal epithelial integrity [7-9]. The NF-κB family consists of five members, p65, RelB, c-Rel, p50 and p52, and forms homo- or heterodimer to regulate transcription [10]. Among them, p65/p50 dimer is essential for TNF-α-induced dysregulation of intestinal epithelial barrier function [11, 12].

Intestinal epithelial cells express several G protein-coupled receptors (GPCRs) including receptors for gastrin, short-chain fatty acids, histamine and acetylcholine [13-17]. Muscarinic acetylcholine receptors have five subtypes, which consisted with $G\alpha_{q/11}$ protein-coupled M1, M3 and M5 receptors and $G\alpha_i$ protein-coupled M2 and M4 receptors. M3 receptor is a major muscarinic subtype expressed in human intestinal epithelium [13, 18]. Muscarinic stimulation induces several important functions including intestinal ion secretion and epithelial cell proliferation. M3 receptors are coupled to phospholipase C (PLC) via $G\alpha_{q/11}$ protein, leading to hydrolysis of phosphatidylinositol bisphosphate and produce diacylglycerol and inositol 1,4,5-trisphosphate, which activate protein kinase C (PKC) and mobilization of intracellular Ca^{2+}, respectively. Recently, it has been reported that $G\alpha_{q/11}$ signalling has a pivotal role to maintain intestinal epithelial homeostasis [19]. Consistent with this, accumulating evidences had shown that M3 receptors could promote protection of epithelial barrier function and prevent colitis development [18, 20, 21]. Histamine H1 receptor is also a $G\alpha_{q/11}$-coupled receptor, which is expressed in intestinal epithelial cells and can induce chloride ion secretion [16, 22]. However, the role of epithelial H1 receptors in intestinal epithelial homeostasis remains unknown. Several GPCRs are known to transactivate EGF receptor (EGFR) through release of EGFR ligands or phosphorylation by non-receptor tyrosine kinases [23-26]. In several epithelial cell lines,

transactivation of EGFR is essential for muscarinic stimulation-mediated activation of ERK1/2 and cell proliferation [27-29]. Furthermore, M3 receptor-mediated activation of EGFR is important to protect intestinal function through the optimal regulation of ion secretion in colon epithelium [30]. Therefore, it is important to elucidate the mechanism of EGFR transactivation for the understanding of M3 receptor-mediated maintenance of intestinal homeostasis.

Previously, we reported that muscarinic stimulation suppressed TNF-α-induced NF-κB signalling and barrier disruption through the shedding of TNFR catalyzed by TNF-α converting enzyme (TACE/ADAM17) [18]. However, it remains unclear what mechanism is involved in M3 receptor-mediated shedding of TNFR and maintenance of epithelial homeostasis. In this study, we showed the functional expression of two $G\alpha_{q/11}$-coupled GPCR, muscarinic M3 and histamine H1 receptors, in HT-29/B6 colonic epithelial cells. Among these receptors, only muscarinic M3 receptor suppressed TNF-α-induced barrier disruption. This protective effect of M3 receptor was mediated by activation of p38 mitogen activating protein kinase (MAPK). Activation of p38 MAPK was also involved in transactivation of EGFR. These results provide the molecular mechanism of M3 receptor-mediated maintenance of intestinal homeostasis.

2. Materials and methods

2.1. Material

Carbachol (CCh) and chlorpheniramine from Sigma Aldrich (MO, USA), fetal bovine serum (FBS) from Invitrogen (CA, USA), recombinant human TNF-α from PEPROTECH (NJ, USA), TNF-α protease inhibitor-0 (TAPI-0) and muscarinic toxin 7 (MT-7) from Peptide Institute (Osaka, Japan). Fura 2-AM from Dojindo (Kumamoto, Japan), AG1478, cimetidine, histamine and YM254890 from Wako pure chemical (Osaka, Japan), darifenacin from Ono Pharmaceutical (Osaka, Japan), SB203580 from AdooQ BioScience (Irvine, CA), recombinant mouse EGF from R&D Systems (Abingdon, UK).

2.2. Cell culture

HT-29/B6 cells are selected from the human colon cancer derived HT-29 cells [31]. HT-29/B6 cells were grown

as highly differentiated polarized epithelial monolayers in a humidified atmosphere with 5% CO2 at 37°C in RPMI 1640 medium supplemented with 2 mM glutamine, 15 mM HEPES (pH 7.2), 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Culture medium was changed every 2 days. For the knockdown experiments, siRNA for TACE gene (HSS110434, Invitrogen) or control siRNA were introduced to the HT-29/B6 cells. Each oligonucleotide was transiently transfected at a final concentration of 10 nM using ScreenFect siRNA (Wako), according to the manufacturer's instruction, and then cells were cultured for 3 days.

2.3. Measurement of intracellular calcium concentration ($[Ca^{2+}]_i$)

HT-29/B6 cells were detached by Pack's D1 solution (5.5 mM glucose, 58.4 mM sucrose, 138 mM NaCl, 5.4 mM KCl, 0.17 mM Na_2HPO_4, and 0.22 mM KH_2PO_4). Then, cells were loaded with 5 μM Fura-2 AM (sonicated and solubilized in 10% Pluronic F-127 just before use) in the growth medium containing 0.7 mM probenecid for 25 min, washed, and then resuspended in Ca^{2+} assay buffer (140 mM NaCl, 5.4 mM KCl, 1.2 mM $MgCl_2$, 2.0 mM $CaCl_2$, 10 mM glucose, and 10 mM HEPES, pH 7.4) with 1 % FBS. $[Ca^{2+}]_i$ was measured by fura-2 ratiofluorometry using a fluorescence spectrophotometer (Hitachi F-4500; Hitachi, Tokyo, Japan). During the measurements, cells were continuously stirred and kept suspended at 37 °C. For inhibitor studies, darifenacin (10 nM), chlorpheniramine (1 μM) or YM254890 (1 μM) were added 3 min before the stimulation of carbachol (100 μM) or histamine (100 μM). The $[Ca^{2+}]_i$ concentration was calculated as previously described [32]. The basal level of $[Ca^{2+}]_i$ was approximately 100 nM, and the net increases in $[Ca^{2+}]_i$ evoked by the drugs were estimated by subtracting $[Ca^{2+}]_i$ of 5-10 sec before drug stimulation from maximum responses.

2.4. Immunoblotting

Cells were stimulated with 100 ng/ml TNF-α for 5 min with or without the pretreatment of carbachol or histamine for 5 min. To check the phosphorylation of MAPKs, cells were maintained in serum free medium for 2 h. Then, cells were stimulated with carbachol, histamine or EGF for 5 min. YM254890, darifenacin, MT-7, chlorpheniramine, cimetidine, SB203580, AG1478 or TAPI-0 was added 15 minutes prior to the stimulation by carbachol. At the end of reaction, the medium was removed and the cells were washed with ice-cold PBS twice. Cells were then lysed by adding a SDS sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 1% SDS, 1% β-mercaptoethanol, 0.1% bromophenol blue). Cell lysates were collected into tubes and heated for 10 minutes at

96°C. Proteins were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA). Membranes were probed with appropriate concentrations of primary antibody against NF-κB p65 (#4764), phospho-NF-κB p65 (Ser536, #3033), IκBα (#4814), phospho-IκBα (Ser32, #2859), p38 MAPK (#9212), phospho-p38 MAPK (Thr180/Tyr182, #4511), ERK1/2 (#4695), phospho-ERK1/2 (Thr202/Tyr204, #4370) (Cell Signaling Technology., MA, USA), β-actin (clone AC-74, Sigma Aldrich, MO, USA) and TACE (ab2051, abcam, MA, USA). The immunoreactive proteins were detected by horseradish-peroxidase-labeled secondary antibody with Clarity Western ECL substrate (BioRad, Hercules, CA). The signal intensity was calculated using Image J software. The phosphorylated levels of NF-κB, IκBα, p38 MAPK and ERK1/2 were normalized to the total levels of NF-κB, β-actin, p38 MAPK and ERK1/2, respectively.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Cells were cultured onto 12-mm Millicell-PCF (Millicell-PCF, 0.4 μm pore size, Millipore, Cork, Ireland) for 9-11 days. After 10 min incubation with fresh medium with or without SB203580, cells were stimulated with carbachol or histamine for 30 min. Then, basolateral medium was collected and assayed by using human sTNFR1 Quantikine ELISA Kit (R&D Systems, Abingdon, UK). Absorbance was measured at 450 nm using a spectrophotometer (SpectraMax PLUS; Molecular Devices, Orleans, CA). sTNFR1 concentration of culture medium without cells was also measured and subtracted. Each value was normalized to the non-stimulated control.

2.6. Measurements of transepithelial resistance (TER)

HT-29/B6 cells were cultured onto 12-mm Millicell-PCF for 9-11 days. TER across the cell monolayers was measured using a Millicell ERS-2 epithelial volt-ohm meter (Millipore, Darmstadt, Germany). The values ($\Omega \times cm^2$) were obtained by subtracting the resistance of blank filters without cells from the resistance of filters with cells multiplied by the effective membrane area of the filter insert. Inserts having TER of at least 600 $\Omega \times cm^2$ was used for the experiments. Carbachol or histamine were added 5 min before the addition of TNF-α. TER was measured at 8, 12 and 24 h after TNF-α treatment. TER at each time was normalized to the initial (0 h) TER.

3. Results

3.1. M3 and H1 receptors stimulate $G\alpha_{q/11}$ dependent Ca^{2+} response

Recently, we showed that muscarinic M3 receptor can suppress TNF-α-induced barrier disruption in intestinal epithelium and human colonic epithelial cell line HT-29/B6 cells [18]. Intestinal mucosal tissue and HT-29 cells also express histamine H1 receptors [16]. Both M3 and H1 receptors can evoke intracellular Ca^{2+} response through $G\alpha_{q/11}$ protein. First, we examined the existence of functional form of M3 and H1 receptors on the cell surface of HT-29/B6 cells through intracellular Ca^{2+} response. Intracellular concentration of Ca^{2+} was measured by fluorometry of Ca^{2+} indicator Fura-2. Just after treatment of carbachol (an acetylcholine analog) or histamine, HT-29/B6 cells showed increase of intracellular Ca^{2+} concentration in a dose-dependent manner (Fig. 1A and B). Each response was suppressed by M3-selective antagonist darifenacin or H1-selective antagonist chlorpheniramine, respectively (Fig. 1C). Furthermore, $G\alpha_{q/11}$ protein inhibitor YM253890 abolished both carbachol- and histamine-induced increases of Ca^{2+} concentration (Fig. 1C). Thus, both M3 and H1 receptors elicit $G\alpha_{q/11}$-mediated Ca^{2+} response in HT-29/B6 cells.

3.2. M3 but not H1 receptor suppresses TNF-α signalling through $G\alpha_{q/11}$ protein

We next examined the role of $G\alpha_{q/11}$ protein in the suppression of TNF-α-induced NF-κB signalling. NF-κB p50/p65 dimer is essential for TNF-α-induced barrier disruption in intestinal epithelial cells [11, 12]. TNF-α induces IκB kinase (IKK)-dependent phosphorylation and proteasomal degradation of IκB. NF-κB p65/p50 dimer, dissociated from IκB complex, translocates into nucleus for the transcription of inflammatory factors. In addition, p65 subunit is phosphorylated for the optimal transcriptional activity [33]. Previously, we reported that TNF-α-induced upregulation of NF-κB transcriptional activity, which was ameliorated by muscarinic stimulation [18]. Activation of M3 receptor inhibits TNF-α-induced phosphorylation of IκB and NF-κB in HT-29/B6 cells. Inhibition of $G\alpha_{q/11}$ protein abolished these M3 receptor-mediated inhibitory effects (Fig. 2A). Thus, $G\alpha_{q/11}$ protein activation is essential for suppression of TNF-α signalling by M3 receptor. Next, we checked the effect of histamine stimulation on TNF-α signalling, because H1 receptor also can evoke $G\alpha_{q/11}$ protein activation in HT-29/B6 cells. Interestingly, histamine stimulation could not ameliorate TNF-α induced NF-κB signalling (Fig. 2B). Because NF-κB activation is required for the TNF-α-induced barrier disruption, TNF-α-induced reduction of TER was

examined with or without muscarinic or histamine stimulation. As shown in Fig. 2C, TNF-α-induced barrier disruption was prevented by muscarinic stimulation but not by histamine. Thus, M3 but not H1 receptor suppresses TNF-α signalling through $G\alpha_{q/11}$ proteins.

3.3. M3 receptor activates p38 MAPK more effectively than H1 receptor

Different effects of M3 and H1 receptors against TNF-α signalling indicate distinct signalling pathways might be activated by each receptor under $G\alpha_{q/11}$ proteins. We recently reported that shedding of TNFR by TACE is required for M3 receptor-mediated suppression of TNF-α signalling [18]. Because p38 MAPK is known to directly upregulate TACE activity, we evaluated p38 MAPK activation by M3 and H1 receptors [34]. Carbachol activated p38 MAPK, which was prevented by darifenacin but not by M1 specific antagonist MT-7 (Fig. 3A). Histamine barely activated p38 MAPK through H1 receptor, although the low phosphorylation was observed (Fig. 3B). The activations of p38 MAPK were completely abolished with YM254890 (Fig. 3C). However, inhibition of $G\alpha_{q/11}$ downstream effector, PKC (10 μM bisindolylmaleimide, BIM), Ca^{2+} response (20 μM BAPTA-AM and 5 mM EGTA) and PLCβ (10 μM U73122) did not affect M3 receptor-mediated p38 MAPK phosphorylation (data not shown). Next, p38 MAPK activation property of M3 and H1 receptors was compared. As shown in Fig. 3D, H1 receptor, compared with M3 receptor, was hardly able to activate p38 MAPK. Thus, M3 receptor but not H1 receptor can effectively activate p38 MAPK through $G\alpha_{q/11}$ protein.

3.4. p38 MAPK activation is crucial for M3 receptor-mediated TNF-α signalling suppression

Because M3 receptor activates p38 MAPK effectively, the role of p38 MAPK on M3 receptor-mediated suppression of TNF-α signalling was examined. As shown in Fig. 4A, the downregulation of TNF-α induced-activation of NF-κB signalling through M3 receptors was ameliorated by p38 MAPK inhibitor SB203580. Previously, we showed the contribution of M3 receptor-induced enhancement of TNF receptor (TNFR) shedding to suppress TNF-α signalling. Carbachol but not histamine stimulation increased the release of soluble TNFR1 (sTNFR1) to the medium. SB203580 suppressed the upregulation of sTNFR1 induced by carbachol (Fig. 4B). Thus, p38 MAPK activation is crucial for M3 receptor-mediated TNFR shedding followed by suppression of TNF-α signalling.

3.5. ERK1/2 activation of M3 receptor is through TACE-mediated EGFR transactivation

M3 receptor can activate MAPK pathways through transactivation of EGFR [29]. EGFR can be activated by GPCR through release of EGFR ligands or phosphorylation of EGFR by src-like kinases. Activation of TACE leads to processing of pro-EGFR ligands including TGF-α, amphiregulin (AR), and heparin-binding EGF (HB-EGF) [35]. As shown in Fig. 5A, M3 receptor-mediated ERK1/2 phosphorylation was abolished by treatment of EGFR inhibitor AG1478. AG1478 showed only partial inhibition of p38 MAPK phosphorylation even in the high concentration. Pharmacological inhibition or siRNA knock-down of TACE also reduced M3 receptor-mediated phosphorylation of ERK1/2, but not that of p38 MAPK (Fig. 5B, 5C). Thus, activation of TACE by M3 receptor also induced ERK1/2 phosphorylation through EGFR transactivation. Transactivation of EGFR was not involved in suppression of TNF-α effects, because inhibition of EGFR did not affect to TNF-α-induced activation of NF-κB signalling (Fig. 5D).

3.6. ERK1/2 activation by M3 receptor is through p38 MAPK activation

Finally, we examined whether M3 receptor-mediated phosphorylation of ERK1/2 was downstream signalling of p38 MAPK activation. Time course of ERK1/2 and p38 MAPK phosphorylation was compared in Fig. 6A. Phosphorylation of ERK1/2 was observed from 2 min after muscarinic stimulation and peaked at 5 min. In contrast, that of p38 MAPK was initiated at 1 min and peaked at 3 min. Thus, phosphorylation of p38 MAPK preceded that of ERK1/2. Next, the effect of p38 MAPK inhibition on ERK1/2 phosphorylation was examined. ERK1/2 phosphorylation by EGF was not affected by SB203580 treatment. On the other hand, SB203580 effectively reduced muscarinic stimulation-induced ERK1/2 phosphorylation in a concentration-dependent manner (Fig. 6B). Phosphorylation of p38 MAPK itself was also inhibited by SB203580. Taken together, these results indicate that p38 MAPK activation by muscarinic stimulation contributes not only to suppression of TNF-α signalling but also to phosphorylation of ERK1/2 through EGFR transactivation.

4. Discussion

In this paper, we demonstrated the existence of M3 and H1 receptors in colonic epithelial cells HT29/B6, however M3 receptor dominantly contributes to the suppression of TNF-α-induced barrier disruption through p38 MAPK activation. p38 MAPK was also involved in TACE-mediated EGFR transactivation followed by ERK1/2 phosphorylation.

First, we demonstrated the existence of functional muscarinic M3 and histamine H1 receptors in HT-29/B6 cells by measurement of intracellular Ca^{2+} response (Fig. 1). Accumulating evidence from *in vivo* and *in vitro* studies has shown that protective role of M3 muscarinic receptors against inflammation in intestinal epithelium [20, 21]. We previously showed that M3 receptors suppress TNF-α-induced barrier disruption through the shedding of TNFR [18]. Wang et al. reported that H1 receptor also induced TNFR shedding in vascular endothelial cells [36]. Both M3 and H1 receptors coupled with $G\alpha_{q/11}$ proteins and can induce PLCβ-dependent increase of intracellular Ca^{2+} concentration and activation of PKC. Suppression of TNF-α-induced NF-κB signalling by muscarinic stimulation was $G\alpha_{q/11}$ protein dependent (Fig. 2A). However, histamine stimulation did not show sufficient reduction of TNF-α signalling and barrier disruption (Fig. 2B, 2C). These results indicate that there is a distinct signalling pathway which is activated particularly by M3 receptors. Concordant with this, p38 MAPK activation was predominantly observed under the muscarinic stimulation (Fig. 3D). Furthermore, inhibition of p38 MAPK with SB203580 abolished M3 receptor-mediated suppression of TNF-α signalling (Fig. 4). Thus, p38 MAPK is crucial to prevent TNF-α-induced barrier disruption by muscarinic stimulation.

It remains unclear how M3, but not H1 receptors, effectively activate p38 MAPK. Signalling pathways evoked by GPCR activation are mainly defined by the G protein coupling specificity. Each GPCR, however, can exert unique signaling pathways, which depends on the environment surrounding the receptors. For instance, oxytocin receptor shows different temporal pattern of EGFR and ERK1/2 activation depending on the localization in caveolar microdomains [37]. AT1 angiotensin II receptor facilitates JAK/STAT signalling through the interaction with JAK2/SHP-2 complex [38]. Likewise, different results of M3 and H1 receptors activation might be ascribed to the distinct microdomains localization or the unique interaction partners. M3 receptor-induced p38 MAPK activation was completely inhibited by specific $G\alpha_{q/11}$ protein inhibitor YM254890. However, inhibition of prototypical $G\alpha_{q/11}$ protein dependent events, PLCβ and PKC activation, and Ca^{2+} response did not influence p38 MAPK phosphorylation (data not shown). Rho-specific guanine nucleotide exchange factor (RhoGEF), p63 RhoGEF and Trio might be candidates of the downstream mediators of $G\alpha_{q/11}$ protein, which could be activated by direct binding

to $G\alpha_q$ protein [39]. Contribution of Rho family GTPases to p38 MAPK activation has been already reported [40]. Interestingly, p38 MAPK inhibitor SB203580 significantly suppressed phosphorylation of p38 MAPK itself (Fig. 6B). p38α MAPK could be activated by autophosphorylation promoted by Tab1, which is independent of MAPKK [41]. Hence, M3 but not H1 receptor-induced $G\alpha_{q/11}$ protein activation might associate with p38 MAPK autophosphorylation machinery.

In this study, we showed that the increase of TACE activity by the M3 receptor-mediated p38 MAPK activation. TACE is responsible protease to produce mature form of TNF-α. In addition, TACE also could suppress TNF-α action through the shedding of TNFR. The shedding of TNFR reduces receptor density and the shedded soluble TNFR intercepts TNF-α, resulting in the attenuation of TNF-α signalling. TACE inhibition in colonic epithelial cells accelerates TNF-α-induced barrier disruption [42]. TACE is known to be activated by various signalling factors, including ROS, NO, PKC and MAPK [43-46]. Previous report had shown that M1 and M3 muscarinic receptors activate TACE and induce prion protein through a PKC dependent fashion [47]. However, our results showed that PKC was not involved in TACE-mediated suppression of TNF-α signalling, because pan-PKC inhibitor BIM showed no effect on this process (data not shown). It has been reported that p38 MAPK directly phosphorylates TACE to activate and induce the surface expression [34, 48]. In this study, we showed that p38 MAPK was a responsive factor for muscarinic stimulation-induced TNFR shedding and TNF-α signalling suppression mediated by TACE. Therefore, p38 MAPK might contribute to protect epithelial cells from TNF-α-induced inflammation. At the same time, p38 MAPK might also induce inflammation through the release of TNF-α from monocytes and macrophages, because, as originally reported, TACE is involved in shedding of pro-TNF-α and release of matured TNF-α [49, 50]. Actually, p38 MAPK can upregulate TACE activity in monocytes [51]. Interestingly, knock-out study indicated that myeloid cell specific deletion of p38α MAPK improved colitis, while intestinal epithelial cells specific deletion of p38α MAPK increased susceptibility to colitis in mouse models of IBD [52]. The differential role of p38α-induced TACE activation, downregulation of TNFR or release of TNF-α, might explain the cell-type dependent role of p38α to the inflammation.

Multiple studies have shown the contribution of EGFR for mucosal homeostasis, which promoted by various effect of EGFR including increase of cell proliferation, suppression of cell extrusion and regulation of ion secretion [53-55]. EGFR can be transactivated by various GPCRs through release of EGFR ligands or phosphorylation by non-receptor tyrosine kinases [23-26]. McCole et al. reported that muscarinic stimulation induced EGFR activation through release of TGF-α and intracellular Src activation in colonic epithelial T84 cells [30]. Our results showed that p38 MAPK activation by muscarinic stimulation induced transactivation of EGFR through TACE activity.

Activation of TACE leads to processing of pro-EGFR ligands including TGF-α, AR, and HB-EGF [35]. Genetic reduction of TACE expression shows increased susceptibility to inflammation in DSS-induced colitis and defective regeneration of epithelial cells, because of impaired shedding of EGFR ligands [56]. Therefore, EGFR transactivation by M3 receptors-mediated TACE activation might also contribute to maintain intestinal epithelial integrity.

In conclusion, our data show muscarinic M3 receptors, but not histamine H1 receptors prevent TNF-α-induced barrier injury through p38 MAPK dependent shedding of TNFR by TACE. p38 MAPK and TACE could be a clinical target for IBD. However, intestinal tissue consisted with variety of cell types including epithelium and myeloid cells. Non-cell specific inhibition of p38 MAPK and TACE might lead to unexpected results, because each factor has cell-dependent role as indicated by several works [52, 56, 57]. Epithelial cell specific regulation of p38 MAPK and TACE through GPCRs, including M3 muscarinic receptors as shown in this study, might provide a practical approach to improve the pharmacological treatment of IBD.

Conflict of interest

The authors declare no conflict of interest.

Grant support

This work was supported in part by JSPS KAKENHI Grant Number 25460378 and 15K10654 (Grant-in-Aid for Scientific Research (C)) and 16K19012 (Grant-in-Aid for Young Scientists (B)) granted by Japan Society for the Promotion of Science, Yamaguchi Endocrine Research Foundation, the fund for Asahikawa Medical University Creative Research in the Field of Life Science and the Smoking Research Foundation of Japan.

Acknowledgements

The authors thank to Ms. S.Tsunoda for the efficient secretarial assistance.

Reference

[1] A. Michielan, R. D'Inca, Intestinal Permeability in Inflammatory Bowel Disease: Pathogenesis, Clinical Evaluation, and Therapy of Leaky Gut, Mediators of inflammation 2015 (2015) 628157.

[2] E.J. Irvine, J.K. Marshall, Increased intestinal permeability precedes the onset of Crohn's disease in a subject with familial risk, Gastroenterology 119(6) (2000) 1740-4.

[3] H. Schmitz, M. Fromm, C.J. Bentzel, P. Scholz, K. Detjen, J. Mankertz, H. Bode, H.J. Epple, E.O. Riecken, J.D. Schulzke, Tumor necrosis factor-alpha (TNFalpha) regulates the epithelial barrier in the human intestinal cell line HT-29/B6, Journal of cell science 112 (Pt 1) (1999) 137-46.

[4] M. Amasheh, I. Grotjohann, S. Amasheh, A. Fromm, J.D. Soderholm, M. Zeitz, M. Fromm, J.D. Schulzke, Regulation of mucosal structure and barrier function in rat colon exposed to tumor necrosis factor alpha and interferon gamma in vitro: a novel model for studying the pathomechanisms of inflammatory bowel disease cytokines, Scandinavian journal of gastroenterology 44(10) (2009) 1226-35.

[5] T.J. Slebioda, Z. Kmiec, Tumour necrosis factor superfamily members in the pathogenesis of inflammatory bowel disease, Mediators of inflammation 2014 (2014) 325129.

[6] U. Billmeier, W. Dieterich, M.F. Neurath, R. Atreya, Molecular mechanism of action of anti-tumor necrosis factor antibodies in inflammatory bowel diseases, World journal of gastroenterology 22(42) (2016) 9300-9313.

[7] A. Oeckinghaus, S. Ghosh, The NF-kappaB family of transcription factors and its regulation, Cold Spring Harbor perspectives in biology 1(4) (2009) a000034.

[8] L. Vereecke, M. Sze, C. Mc Guire, B. Rogiers, Y. Chu, M. Schmidt-Supprian, M. Pasparakis, R. Beyaert, G. van Loo, Enterocyte-specific A20 deficiency sensitizes to tumor necrosis factor-induced toxicity and experimental colitis, The Journal of experimental medicine 207(7) (2010) 1513-23.

[9] K. Vlantis, A. Wullaert, Y. Sasaki, M. Schmidt-Supprian, K. Rajewsky, T. Roskams, M. Pasparakis, Constitutive IKK2 activation in intestinal epithelial cells induces intestinal tumors in mice, The Journal of clinical investigation 121(7) (2011) 2781-93.

[10] Q. Zhang, M.J. Lenardo, D. Baltimore, 30 Years of NF-kappaB: A Blossoming of Relevance to Human Pathobiology, Cell 168(1-2) (2017) 37-57.

[11] D. Ye, I. Ma, T.Y. Ma, Molecular mechanism of tumor necrosis factor-alpha modulation of intestinal epithelial tight junction barrier, American journal of physiology. Gastrointestinal and liver physiology 290(3) (2006) G496-504.

[12] R. Al-Sadi, S. Guo, D. Ye, M. Rawat, T.Y. Ma, TNF-alpha Modulation of Intestinal Tight Junction Permeability Is

Mediated by NIK/IKK-alpha Axis Activation of the Canonical NF-kappaB Pathway, The American journal of pathology 186(5) (2016) 1151-65.

[13] H. Frucht, R.T. Jensen, D. Dexter, W.L. Yang, Y. Xiao, Human colon cancer cell proliferation mediated by the M3 muscarinic cholinergic receptor, Clinical cancer research : an official journal of the American Association for Cancer Research 5(9) (1999) 2532-9.

[14] M.R. Khan, A.S. Anisuzzaman, S. Semba, Y. Ma, J. Uwada, H. Hayashi, Y. Suzuki, T. Takano, H. Ikeuchi, M. Uchino, A. Maemoto, F. Ushikubi, I. Muramatsu, T. Taniguchi, M1 is a major subtype of muscarinic acetylcholine receptors on mouse colonic epithelial cells, Journal of gastroenterology 48(8) (2013) 885-96.

[15] R. Colucci, C. Blandizzi, M. Tanini, C. Vassalle, M.C. Breschi, M. Del Tacca, Gastrin promotes human colon cancer cell growth via CCK-2 receptor-mediated cyclooxygenase-2 induction and prostaglandin E2 production, British journal of pharmacology 144(3) (2005) 338-48.

[16] F. Cianchi, C. Cortesini, N. Schiavone, F. Perna, L. Magnelli, E. Fanti, D. Bani, L. Messerini, V. Fabbroni, G. Perigli, S. Capaccioli, E. Masini, The role of cyclooxygenase-2 in mediating the effects of histamine on cell proliferation and vascular endothelial growth factor production in colorectal cancer, Clinical cancer research : an official journal of the American Association for Cancer Research 11(19 Pt 1) (2005) 6807-15.

[17] H. Tazoe, Y. Otomo, I. Kaji, R. Tanaka, S.I. Karaki, A. Kuwahara, Roles of short-chain fatty acids receptors, GPR41 and GPR43 on colonic functions, Journal of physiology and pharmacology : an official journal of the Polish Physiological Society 59 Suppl 2 (2008) 251-62.

[18] M.R. Khan, J. Uwada, T. Yazawa, M.T. Islam, S.M. Krug, M. Fromm, S. Karaki, Y. Suzuki, A. Kuwahara, H. Yoshiki, K. Sada, I. Muramatsu, A.S. Anisuzzaman, T. Taniguchi, Activation of muscarinic cholinoceptor ameliorates tumor necrosis factor-alpha-induced barrier dysfunction in intestinal epithelial cells, FEBS letters 589(23) (2015) 3640-7.

[19] N. Watanabe, H. Mashima, K. Miura, T. Goto, M. Yoshida, A. Goto, H. Ohnishi, Requirement of Ga_q/Ga_{11} signaling in the preservation of mouse intestinal epithelial homeostasis, Cellular and Molecular Gastroenterology and Hepatology 2(6) (2016) 767-782.e6.

[20] S. Dhawan, I.H. Hiemstra, C. Verseijden, F.W. Hilbers, A.A. Te Velde, L.E. Willemsen, J. Stap, J.M. den Haan, W.J. de Jonge, Cholinergic receptor activation on epithelia protects against cytokine-induced barrier dysfunction, Acta physiologica 213(4) (2015) 846-59.

[21] C.L. Hirota, D.M. McKay, M3 muscarinic receptor-deficient mice retain bethanechol-mediated intestinal ion transport and are more sensitive to colitis, Canadian journal of physiology and pharmacology 84(11) (2006) 1153-61.

[22] G. Schultheiss, B. Hennig, W. Schunack, G. Prinz, M. Diener, Histamine-induced ion secretion across rat distal colon: involvement of histamine H1 and H2 receptors, European journal of pharmacology 546(1-3) (2006) 161-70.

[23] H.L. Hsieh, C.C. Lin, H.J. Chan, C.M. Yang, C.M. Yang, c-Src-dependent EGF receptor transactivation contributes to ET-1-induced COX-2 expression in brain microvascular endothelial cells, Journal of neuroinflammation 9 (2012) 152.

[24] L.J. Watson, K.M. Alexander, M.L. Mohan, A.L. Bowman, S. Mangmool, K. Xiao, S.V. Naga Prasad, H.A. Rockman, Phosphorylation of Src by phosphoinositide 3-kinase regulates beta-adrenergic receptor-mediated EGFR transactivation, Cellular signalling 28(10) (2016) 1580-92.

[25] P.J. Little, M.D. Hollenberg, D. Kamato, W. Thomas, J. Chen, T. Wang, W. Zheng, N. Osman, Integrating the GPCR transactivation-dependent and biased signalling paradigms in the context of PAR1 signalling, British journal of pharmacology 173(20) (2016) 2992-3000.

[26] F. Cattaneo, G. Guerra, M. Parisi, M. De Marinis, D. Tafuri, M. Cinelli, R. Ammendola, Cell-surface receptors transactivation mediated by g protein-coupled receptors, Int J Mol Sci 15(11) (2014) 19700-28.

[27] H. Yu, H. Xia, Q. Tang, H. Xu, G. Wei, Y. Chen, X. Dai, Q. Gong, F. Bi, Acetylcholine acts through M3 muscarinic receptor to activate the EGFR signaling and promotes gastric cancer cell proliferation, Scientific reports 7 (2017) 40802.

[28] W. Ockenga, S. Kuhne, S. Bocksberger, A. Banning, R. Tikkanen, Epidermal growth factor receptor transactivation is required for mitogen-activated protein kinase activation by muscarinic acetylcholine receptors in HaCaT keratinocytes, Int J Mol Sci 15(11) (2014) 21433-54.

[29] K. Cheng, P. Zimniak, J.P. Raufman, Transactivation of the epidermal growth factor receptor mediates cholinergic agonist-induced proliferation of H508 human colon cancer cells, Cancer research 63(20) (2003) 6744-50.

[30] D.F. McCole, S.J. Keely, R.J. Coffey, K.E. Barrett, Transactivation of the epidermal growth factor receptor in colonic epithelial cells by carbachol requires extracellular release of transforming growth factor-alpha, J Biol Chem 277(45) (2002) 42603-12.

[31] K.M. Kreusel, M. Fromm, J.D. Schulzke, U. Hegel, Cl- secretion in epithelial monolayers of mucus-forming human colon cells (HT-29/B6), The American journal of physiology 261(4 Pt 1) (1991) C574-82.

[32] H. Yoshiki, J. Uwada, H. Umada, T. Kobayashi, T. Takahashi, T. Yamakawa, A. Yamaguchi, O. Yokoyama, I. Muramatsu, Agonist pharmacology at recombinant alpha1A - and alpha1L -adrenoceptors and in lower urinary tract alpha1 -adrenoceptors, British journal of pharmacology 170(6) (2013) 1242-52.

[33] P. Viatour, M.P. Merville, V. Bours, A. Chariot, Phosphorylation of NF-kappaB and IkappaB proteins: implications in cancer and inflammation, Trends in biochemical sciences 30(1) (2005) 43-52.

[34] P. Xu, R. Derynck, Direct activation of TACE-mediated ectodomain shedding by p38 MAP kinase regulates EGF receptor-dependent cell proliferation, Molecular cell 37(4) (2010) 551-66.

[35] D.C. Lee, S.W. Sunnarborg, C.L. Hinkle, T.J. Myers, M.Y. Stevenson, W.E. Russell, B.J. Castner, M.J. Gerhart, R.J. Paxton, R.A. Black, A. Chang, L.F. Jackson, TACE/ADAM17 processing of EGFR ligands indicates a role as a physiological convertase, Ann N Y Acad Sci 995 (2003) 22-38.

[36] J. Wang, R.S. Al-Lamki, H. Zhang, N. Kirkiles-Smith, M.L. Gaeta, S. Thiru, J.S. Pober, J.R. Bradley, Histamine antagonizes tumor necrosis factor (TNF) signaling by stimulating TNF receptor shedding from the cell surface and Golgi storage pool, J Biol Chem 278(24) (2003) 21751-60.

[37] V. Rimoldi, A. Reversi, E. Taverna, P. Rosa, M. Francolini, P. Cassoni, M. Parenti, B. Chini, Oxytocin receptor elicits different EGFR/MAPK activation patterns depending on its localization in caveolin-1 enriched domains, Oncogene 22(38) (2003) 6054-60.

[38] M.B. Marrero, V.J. Venema, H. Ju, D.C. Eaton, R.C. Venema, Regulation of angiotensin II-induced JAK2 tyrosine phosphorylation: roles of SHP-1 and SHP-2, The American journal of physiology 275(5 Pt 1) (1998) C1216-23.

[39] R.J. Rojas, M.E. Yohe, S. Gershburg, T. Kawano, T. Kozasa, J. Sondek, Galphaq directly activates p63RhoGEF and Trio via a conserved extension of the Dbl homology-associated pleckstrin homology domain, J Biol Chem 282(40) (2007) 29201-10.

[40] J.P. Vaque, R.T. Dorsam, X. Feng, R. Iglesias-Bartolome, D.J. Forsthoefel, Q. Chen, A. Debant, M.A. Seeger, B.R. Ksander, H. Teramoto, J.S. Gutkind, A genome-wide RNAi screen reveals a Trio-regulated Rho GTPase circuitry transducing mitogenic signals initiated by G protein-coupled receptors, Molecular cell 49(1) (2013) 94-108.

[41] B. Ge, H. Gram, F. Di Padova, B. Huang, L. New, R.J. Ulevitch, Y. Luo, J. Han, MAPKK-independent activation of p38alpha mediated by TAB1-dependent autophosphorylation of p38alpha, Science 295(5558) (2002) 1291-4.

[42] T. Freour, A. Jarry, K. Bach-Ngohou, T. Dejoie, C. Bou-Hanna, M.G. Denis, J.F. Mosnier, C.L. Laboisse, D. Masson, TACE inhibition amplifies TNF-alpha-mediated colonic epithelial barrier disruption, International journal of molecular medicine 23(1) (2009) 41-8.

[43] T.J. Myers, L.H. Brennaman, M. Stevenson, S. Higashiyama, W.E. Russell, D.C. Lee, S.W. Sunnarborg, Mitochondrial reactive oxygen species mediate GPCR-induced TACE/ADAM17-dependent transforming growth factor-alpha shedding, Molecular biology of the cell 20(24) (2009) 5236-49.

[44] E. Diaz-Rodriguez, J.C. Montero, A. Esparis-Ogando, L. Yuste, A. Pandiella, Extracellular signal-regulated kinase phosphorylates tumor necrosis factor alpha-converting enzyme at threonine 735: a potential role in regulated shedding, Molecular biology of the cell 13(6) (2002) 2031-44.

[45] Z. Zhang, J.K. Kolls, P. Oliver, D. Good, P.O. Schwarzenberger, M.S. Joshi, J.L. Ponthier, J.R. Lancaster, Jr., Activation of tumor necrosis factor-alpha-converting enzyme-mediated ectodomain shedding by nitric oxide, J Biol Chem 275(21) (2000) 15839-44.

[46] B. Vincent, E. Paitel, P. Saftig, Y. Frobert, D. Hartmann, B. De Strooper, J. Grassi, E. Lopez-Perez, F. Checler, The disintegrins ADAM10 and TACE contribute to the constitutive and phorbol ester-regulated normal cleavage of the cellular prion protein, J Biol Chem 276(41) (2001) 37743-6.

[47] M. Alfa Cisse, C. Sunyach, B.E. Slack, A. Fisher, B. Vincent, F. Checler, M1 and M3 muscarinic receptors control physiological processing of cellular prion by modulating ADAM17 phosphorylation and activity, J Neurosci 27(15) (2007) 4083-92.

[48] P. Xu, J. Liu, M. Sakaki-Yumoto, R. Derynck, TACE activation by MAPK-mediated regulation of cell surface dimerization and TIMP3 association, Science signaling 5(222) (2012) ra34.

[49] R.A. Black, C.T. Rauch, C.J. Kozlosky, J.J. Peschon, J.L. Slack, M.F. Wolfson, B.J. Castner, K.L. Stocking, P. Reddy, S. Srinivasan, N. Nelson, N. Boiani, K.A. Schooley, M. Gerhart, R. Davis, J.N. Fitzner, R.S. Johnson, R.J. Paxton, C.J. March, D.P. Cerretti, A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells, Nature 385(6618) (1997) 729-33.

[50] M.L. Moss, S.L. Jin, M.E. Milla, D.M. Bickett, W. Burkhart, H.L. Carter, W.J. Chen, W.C. Clay, J.R. Didsbury, D. Hassler, C.R. Hoffman, T.A. Kost, M.H. Lambert, M.A. Leesnitzer, P. McCauley, G. McGeehan, J. Mitchell, M. Moyer, G. Pahel, W. Rocque, L.K. Overton, F. Schoenen, T. Seaton, J.L. Su, J.D. Becherer, et al., Cloning of a disintegrin metalloproteinase that processes precursor tumour-necrosis factor-alpha, Nature 385(6618) (1997) 733-6.

[51] A.J. Scott, K.P. O'Dea, D. O'Callaghan, L. Williams, J.O. Dokpesi, L. Tatton, J.M. Handy, P.J. Hogg, M. Takata, Reactive oxygen species and p38 mitogen-activated protein kinase mediate tumor necrosis factor alpha-converting enzyme (TACE/ADAM-17) activation in primary human monocytes, J Biol Chem 286(41) (2011) 35466-76.

[52] M. Otsuka, Y.J. Kang, J. Ren, H. Jiang, Y. Wang, M. Omata, J. Han, Distinct effects of p38alpha deletion in myeloid lineage and gut epithelia in mouse models of inflammatory bowel disease, Gastroenterology 138(4) (2010) 1255-65, 1265 e1-9.

[53] P.E. Dube, F. Yan, S. Punit, N. Girish, S.J. McElroy, M.K. Washington, D.B. Polk, Epidermal growth factor receptor inhibits colitis-associated cancer in mice, The Journal of clinical investigation 122(8) (2012) 2780-92.

[54] D.F. McCole, K.E. Barrett, Decoding epithelial signals: critical role for the epidermal growth factor receptor in controlling intestinal transport function, Acta physiologica 195(1) (2009) 149-59.

[55] J.C. Miguel, A.A. Maxwell, J.J. Hsieh, L.C. Harnisch, D. Al Alam, D.B. Polk, C.L. Lien, A.J. Watson, M.R. Frey, Epidermal growth factor suppresses intestinal epithelial cell shedding through a MAPK-dependent pathway, Journal of cell science 130(1) (2017) 90-96.

[56] A. Chalaris, N. Adam, C. Sina, P. Rosenstiel, J. Lehmann-Koch, P. Schirmacher, D. Hartmann, J. Cichy, O. Gavrilova, S. Schreiber, T. Jostock, V. Matthews, R. Hasler, C. Becker, M.F. Neurath, K. Reiss, P. Saftig, J. Scheller, S. Rose-John,

Critical role of the disintegrin metalloprotease ADAM17 for intestinal inflammation and regeneration in mice, The Journal of experimental medicine 207(8) (2010) 1617-24.

[57] K. Horiuchi, T. Kimura, T. Miyamoto, H. Takaishi, Y. Okada, Y. Toyama, C.P. Blobel, Cutting edge: TNF-alpha-converting enzyme (TACE/ADAM17) inactivation in mouse myeloid cells prevents lethality from endotoxin shock, Journal of immunology 179(5) (2007) 2686-9.

Figure legends

Fig. 1. M3 and H1 receptors stimulate $G\alpha_{q/11}$ dependent Ca^{2+} response.

(A) Representative traces of the increases in $[Ca^{2+}]_i$ from the basal level, in response to carbachol (100 μM) or histamine (100 μM) in HT-29/B6 cells. Each agonist was administrated at 20 s as indicated by the arrow. (B) HT-29/B6 cells were treated with different concentration of carbachol or histamine. The maximal increases in $[Ca^{2+}]_i$ from the basal level were plotted. n = 3-6 for each condition. (C) The histogram shows the maximal increases in $[Ca^{2+}]_i$ from the basal level. Carbachol or histamine (100 μM each) was administrated in the absence or presence of muscarinic M3 receptor selective antagonist darifenacin (10 nM), histamine H1 selective antagonist chlorpheniramine (1 μM) or $G\alpha_{q/11}$ inhibitor YM254890 (1 μM). *p < 0.05, **p < 0.01, when compared with carbachol or histamine alone (one-way ANOVA with Dunnett's post hoc test). Values represent the means ± S.E.M. (n = 3-4 for each condition).

Fig. 2. M3 but not H1 receptor suppresses TNF-α signalling through $G\alpha_{q/11}$ protein.

(A) (B) HT-29/B6 cells were treated with TNF-α for 5 minutes with or without the pretreatment of carbachol (CCh, 100 μM) and YM254890 (1 μM) (A) or indicated concentration of carbachol and histamine (B). Cell lysates were subjected to immunoblot by using phosphorylated NF-κB, NF-κB, phosphorylated IκB, IκB, and β-actin antibodies. The ratio of intensities of signals was quantified by densitometry (right). (A) *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (one-way ANOVA with Tukey's post hoc test). Values represent the means ± S.E.M. of 4 independent experiments. (B) **$p < 0.01$, ***$p < 0.001$ when compared with TNF-α alone (one-way ANOVA with Dunnett's post hoc test). n.s., not significantly different. Values represent the means ± S.E.M. of 4 independent experiments. (C) TER was measured at 8, 12 and 24 h after TNF-α treatment with or without carbachol or histamine. TER at each time was normalized to the initial (0h) TER. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ when compared with TNF-α alone (one-way ANOVA with Dunnett's post hoc test). Values represent the means ± S.E.M. of 4 independent experiments.

Fig. 3. M3 receptor activates p38 MAPK more effectively than H1 receptor.

(A) HT-29/B6 cells were treated with carbachol (100 μM) for 5 minutes with or without the pretreatment of M1 specific antagonist MT-7 (100 nM) or indicated concentration of darifenacin. Cell lysates were subjected to immunoblot by using phosphorylated p38 MAPK and p38 MAPK antibodies. (B) HT-29/B6 cells were treated with histamine (100 μM) for 5 minutes with or without the pretreatment of indicated concentration of chlorpheniramine

or histamine H2 receptor blocker cimetidine. (C) HT-29/B6 cells were treated with carbachol (100 μM) or histamine (100 μM) with or without YM254890 (1 μM). (D) HT-29/B6 cells were treated with the indicated concentration of carbachol or histamine. The ratio of intensities of signal was quantified by densitometry (bellow). Values represent the means ± S.E.M. of 3 independent experiments.

Fig. 4. p38 MAPK activation is crucial for M3 receptor-mediated TNF-α signalling suppression.

(A) HT-29/B6 cells were treated with TNF-α for 5 minutes with or without the pretreatment of carbachol (CCh, 100 μM) and SB203580 (1 μM). The ratio of intensities of signal was quantified by densitometry (right). $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (one-way ANOVA with Tukey's post hoc test). Values represent the means ± S.E.M. of 5 independent experiments. (B) HT-29/B6 cell monolayers were treated with carbachol (10, 100 μM), histamine (100 μM) and SB203580 (1 μM). After 30 min incubation, the basolateral medium was collected and applied to ELISA kit for sTNFR1. Each value was normalized to control. $*p < 0.05$, $***p < 0.001$, when compared with control (one-way ANOVA with Dunnett's post hoc test). n = 3-9 for each condition. Values represent the means ± S.E.M.

Fig. 5. ERK1/2 activation of M3 receptor is through TACE-mediated EGFR transactivation.

(A) (B) HT-29/B6 cells were treated with carbachol (100 μM) for 5 minutes with or without the pretreatment of the indicated concentration of EGFR tyrosine kinase inhibitor AG1478 (A) or TACE inhibitor TAPI-0 (B). Cell lysates were subjected to immunoblot by using phosphorylated ERK1/2, ERK1/2, phosphorylated p38 MAPK and p38 MAPK antibodies. (C) HT-29/B6 cells were incubated with TACE-targeted siRNA for 3 days. Then cells were stimulated with carbachol (100 μM) for 5 min. The ratio of intensities of signal was quantified by densitometry (right). $***p < 0.001$ (Student's t test). n.s., not significantly different. Values represent the means ± S.E.M. of 3 independent experiments. (D) HT-29/B6 cells were treated with TNF-α for 5 minutes with or without the pretreatment of carbachol (CCh, 100 μM) and AG1478 (300 nM). The ratio of intensities of signal was quantified by densitometry (right). $**p < 0.01$, $***p < 0.001$ (one-way ANOVA with Tukey's post hoc test). n.s., not significantly different. Values represent the means ± S.E.M. of 4 independent experiments.

Fig. 6. ERK1/2 activation of M3 receptor is through p38 MAPK activation.

(A) HT-29/B6 cells were treated with carbachol (100 μM) for the indicated periods. Time courses of ERK1/2 and p38 MAPK phosphorylation were plotted on the bellow graph. (B) HT-29/B6 cells were treated with carbachol (100 μM) or EGF (20 ng/ml) with or without the indicated concentration of SB203580. The ratio of quantified

phosphorylated ERK1/2 signals was represented in a graph. *$p < 0.05$, ***$p < 0.001$ when compared with carbachol alone (one-way ANOVA with Dunnett's post hoc test). n.s., not significantly different (Student's t test). Values represent the means ± S.E.M. of 4 independent experiments.

Figure(s)

Fig. 1

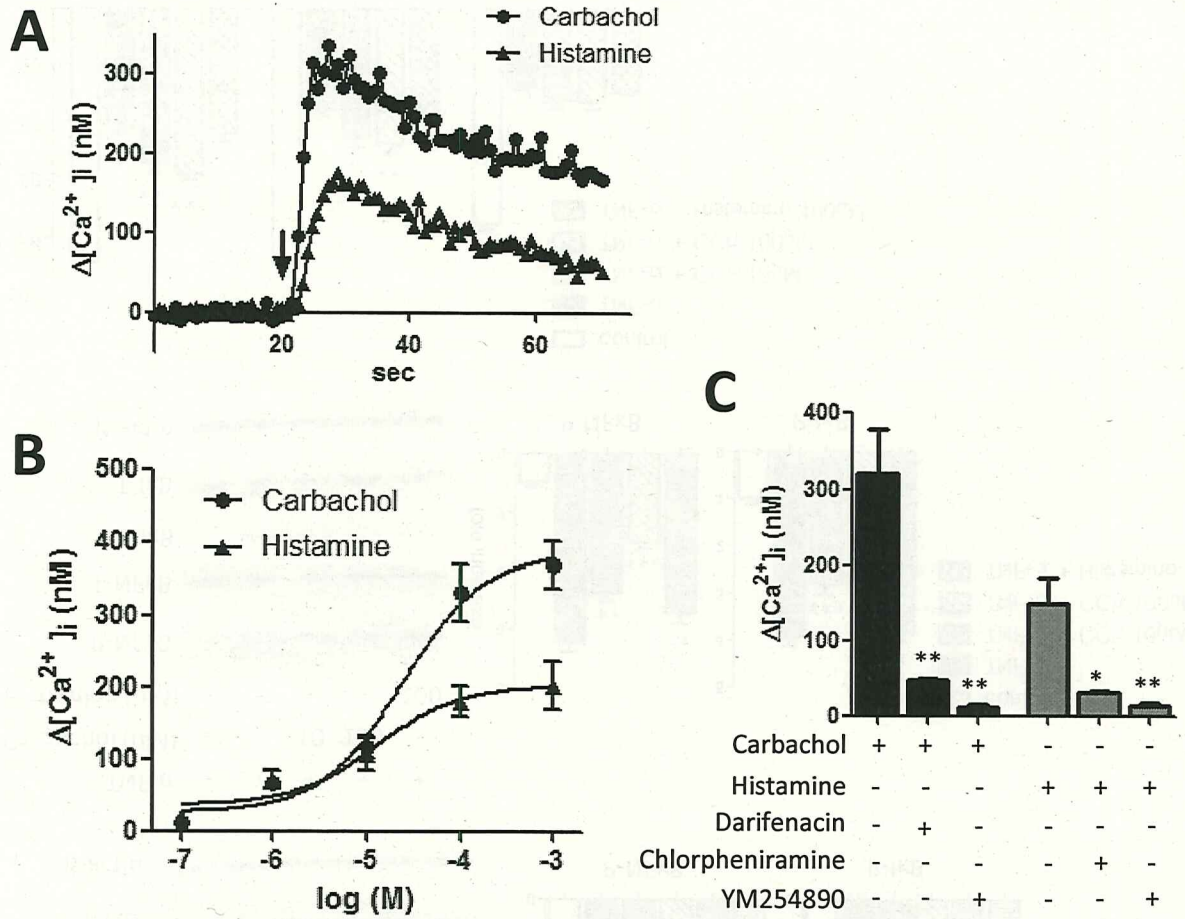

Fig. 2

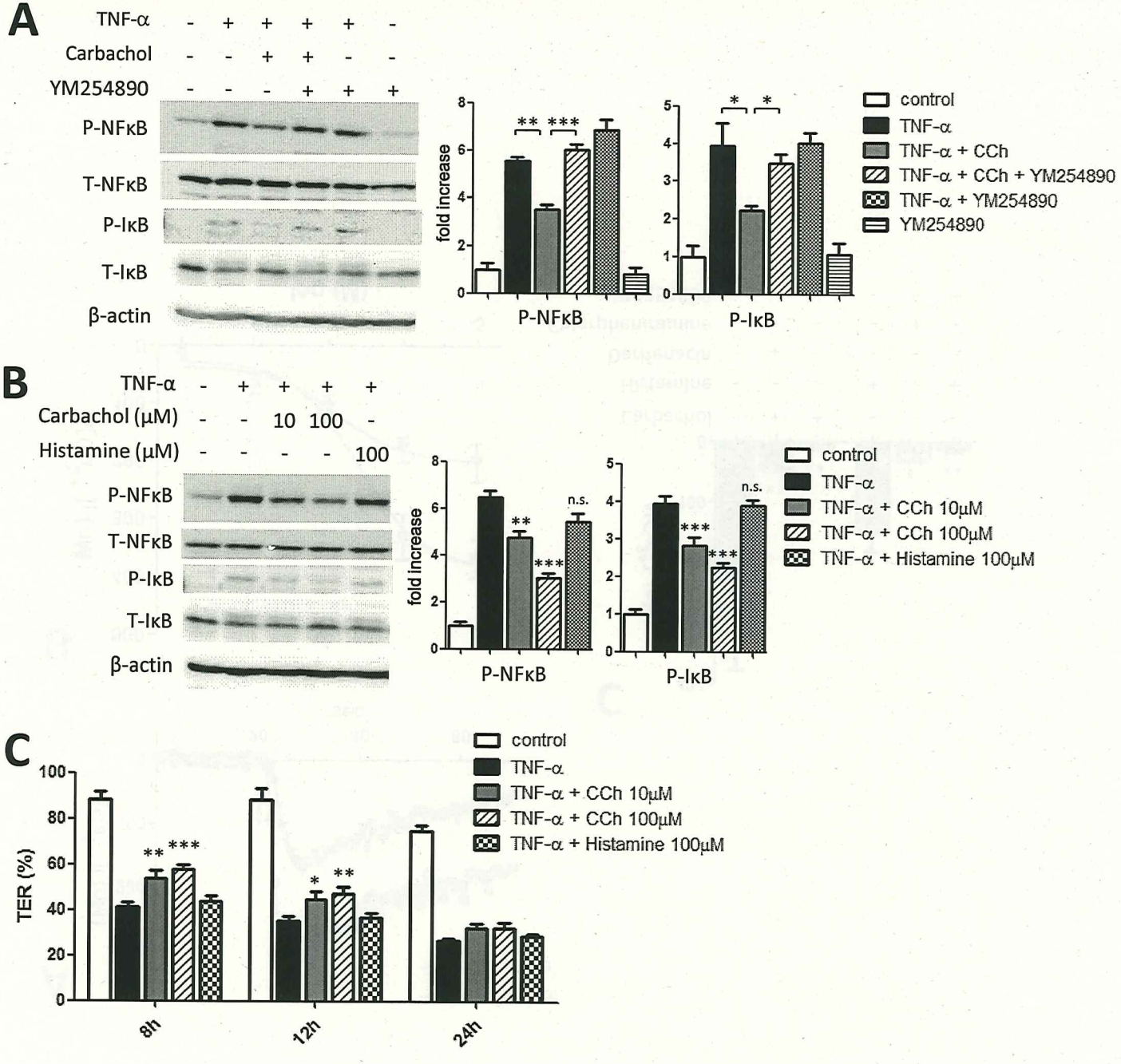
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Fig. 3

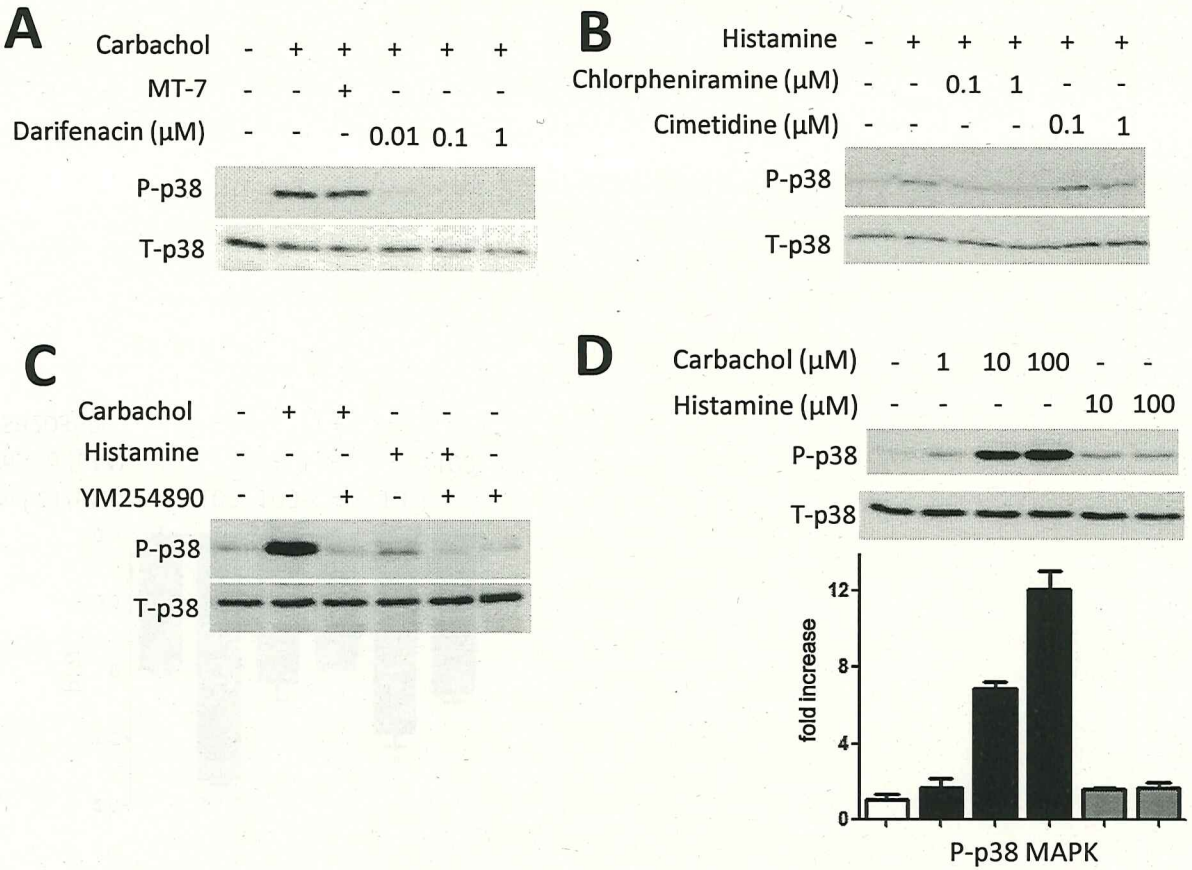
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Fig. 4

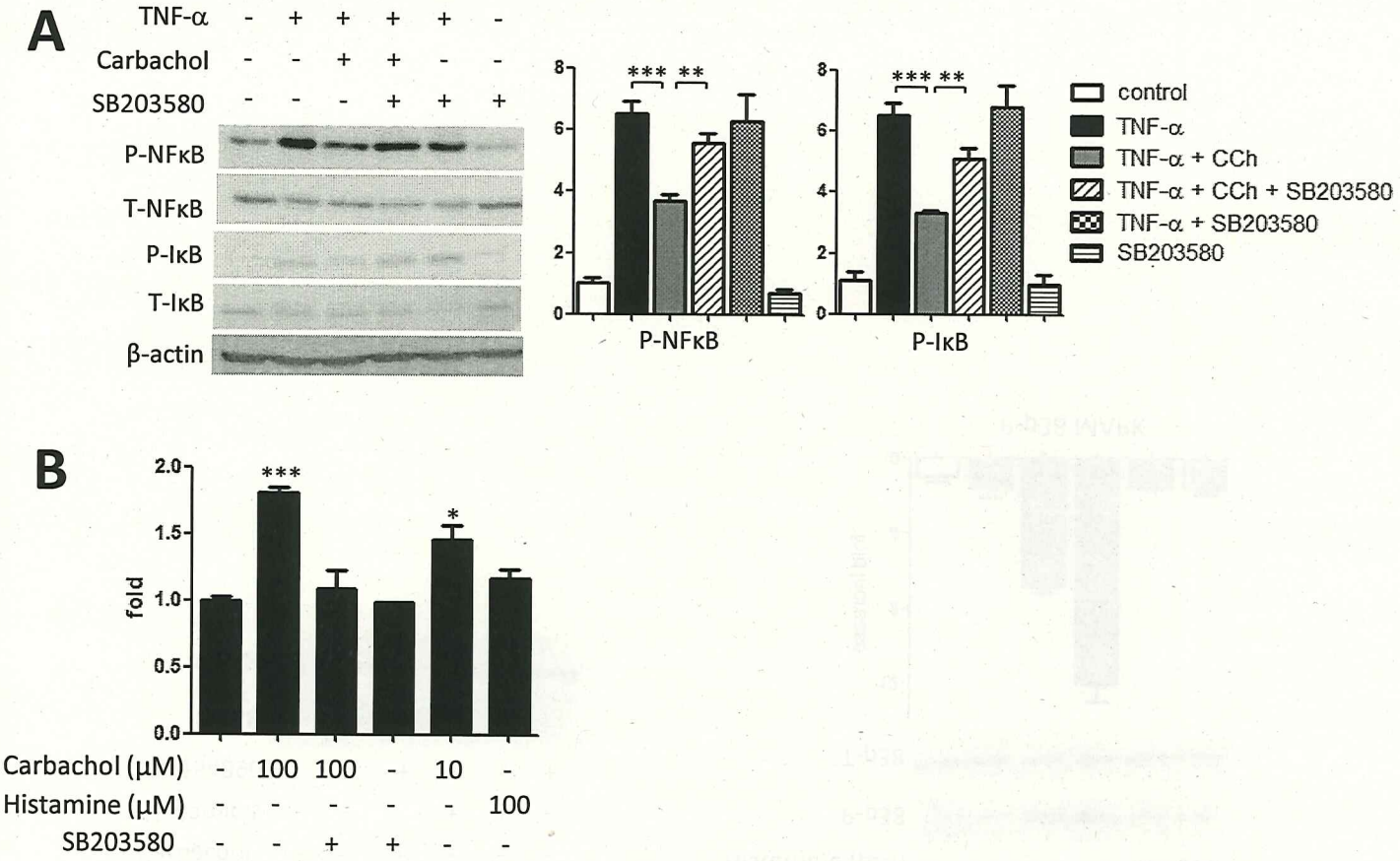
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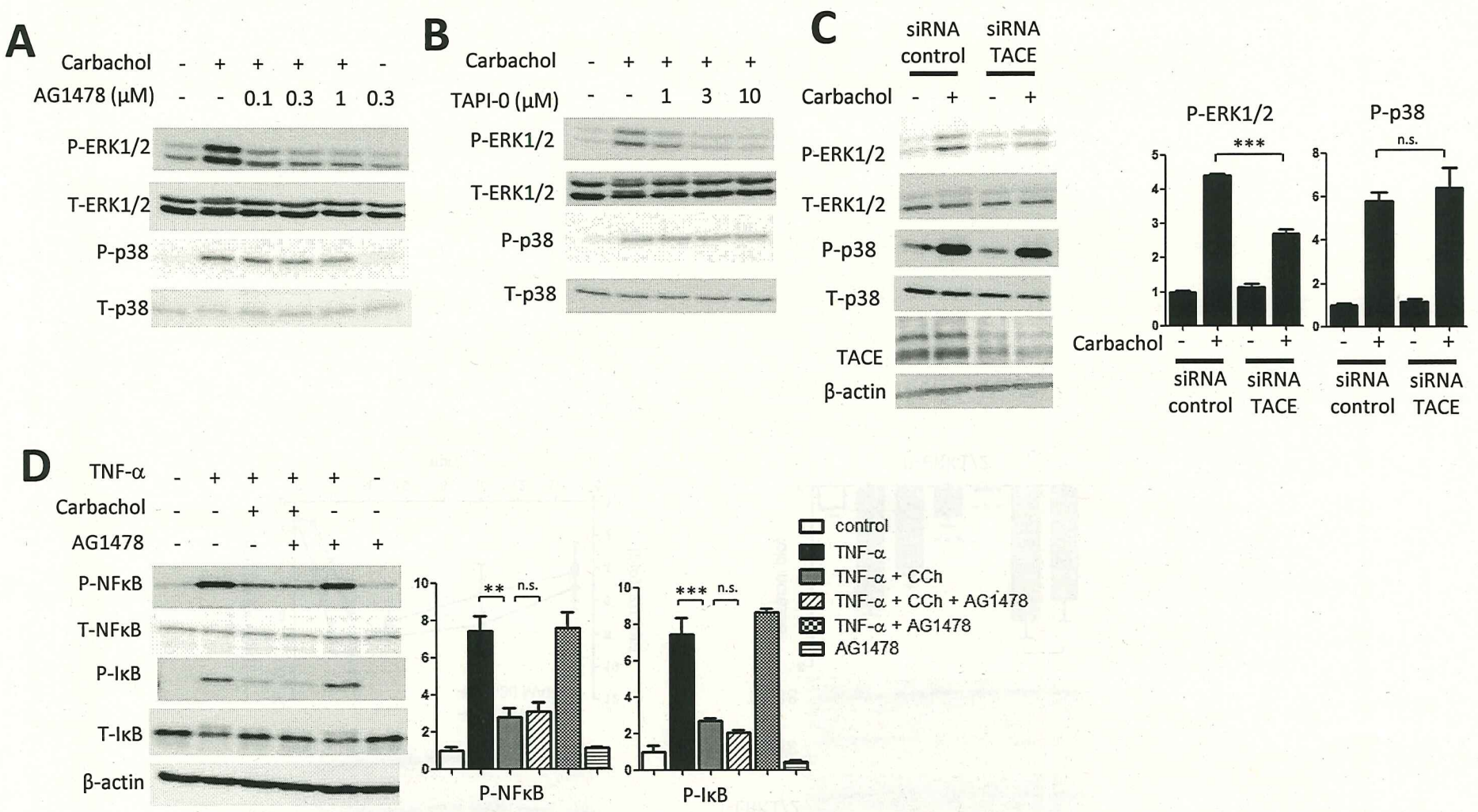
Fig. 5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P-IκB
T-IκB
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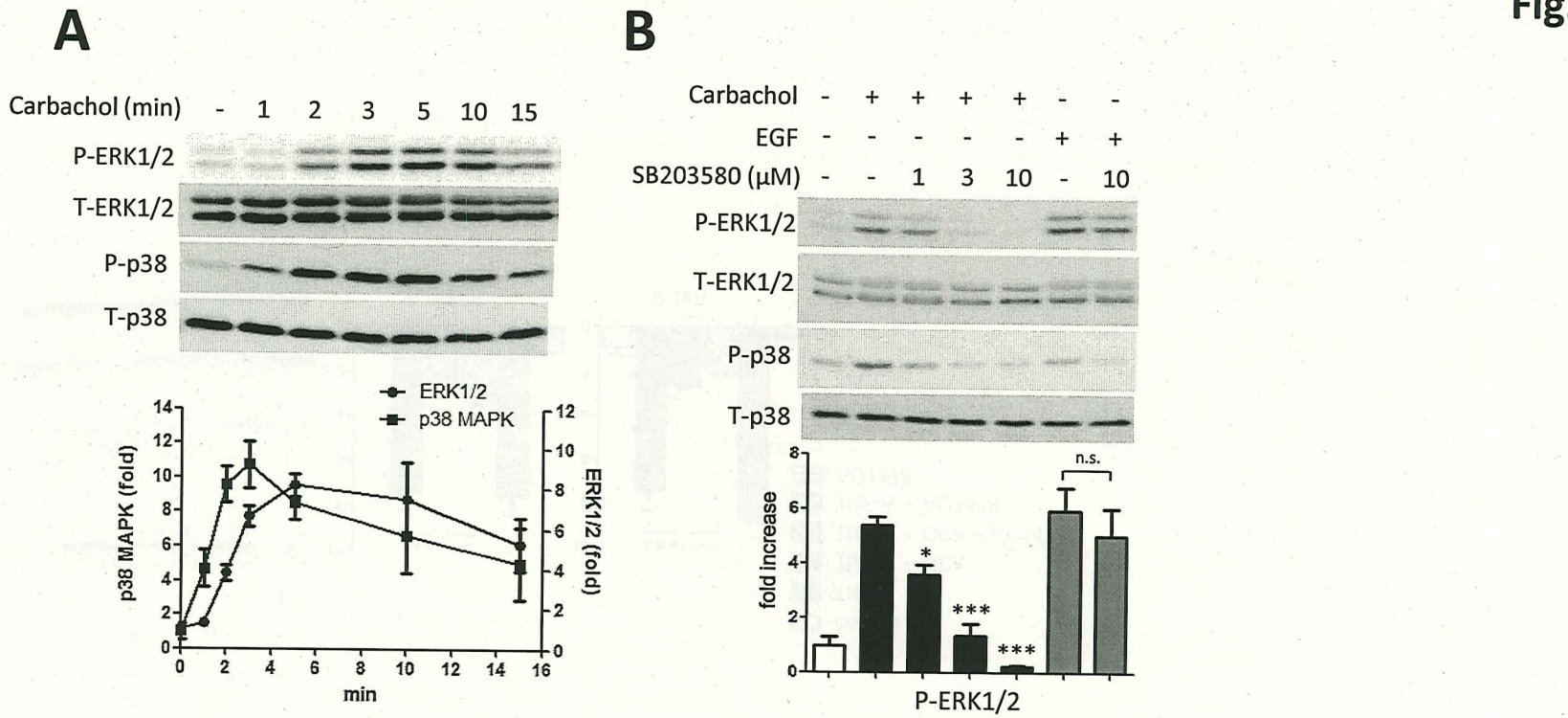
P-IκB

Fig. 6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P-p38
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EGF - - - - - + +
SB203580 (μM) - - 1 3 10 - 10
P-ERK1/2
T-ERK1/2
P-p38
T-p38
fold increase
n.s.
*
***
***
P-ERK1/2