Molecular Pharmacology Fast Forward. Published on April 17, 2012 as doi:10.1124/mol.111.077354

MOL #77354

Muscarinic type 3 receptor induces cytoprotective signaling in salivary gland cells via EGFR transactivation

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Molecular Pharmacology Fast Forward. Published on April 17, 2012 as DOI: 10.1124/mol.111.077354
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Running title: M3R mediates salivary gland cell survival

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Text pages: 46

Tables: 0

Figures: 8

References: 50

Words in the Abstract: 250

Words in the Introduction: 649

Words in the Discussion: 1499

Abbreviations:

ERK, extracellular signal-regulated kinase; EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; siRNA, small interfering RNA; HRP, horseradish peroxidase; DMEM. Dulbecco's modified eagle medium; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide;

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Abstract

Muscarinic type 3 receptor (M3R) plays a pivotal role in the induction of glandular fluid secretions. While M3R is often the target of autoantibody in Sjögren's syndrome (SjS), chemical agonists for M3R are clinically used to stimulate the saliva secretion in SjS patients. However, aside from its activity in promoting glandular fluid secretions, it is unclear whether activation of M3R is related to other biological events in SjS. This study aimed to investigate the cytoprotective effect of chemical-agonist-mediated M3R-activation on apoptosis induced in human salivary gland (HSG) cells. Carbachol (CCh), a muscarinic receptor-specific agonist, abrogated TNFα/IFNγ-induced apoptosis via pathways involving caspase3/7, but its cytoprotective effect was diminished by M3R antagonist (4-DAMP), MEK-ERK inhibitor (U0126), PI3K-Akt inhibitor (LY294002), or EGFR inhibitor (AG1478). Ligation of M3R with CCh transactivated EGFR and phosphorylated ERK and Akt, the downstream target of EGFR. Inhibition of intracellular calcium release or PKCδ, both of which are involved in the cell signaling of M3R-mediated fluid secretion, did not affect CCh-induced ERK- or Akt-phosphorylation. On the other hand, CCh stimulated the phosphorylation of Src and Src-binding to EGFR. Importantly, Src inhibitor (PP2) attenuated the CCh/M3R-induced cytoprotective effect and EGFR transactivation cascades. Overall, these results indicated that

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CCh/M3R induced transactivation of EGFR via Src activation, leading to ERK- and Akt-phosphorylation which, in turn, suppressed caspase3/7-mediated apoptotic signals in HSG cells. Therefore, this study, for the first time, proposes that CCh-mediated M3R activation can promote not only fluid secretion but also survival of salivary gland cells in the inflammatory context of SjS.

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Introduction

Sjögren's syndrome (SjS) is a chronic autoimmune disease characterized by lymphocytic infiltration, gland destruction, along with eye and mouth dryness (Fox and Kang, 1992; Fox and Stern, 2002; Kroneld et al., 1997). Although the etiology of SjS remains unclear, many studies have suggested that T and B lymphocytes that infiltrate the affected glands are engaged in the pathogenesis of SjS because of their production of tissue-destructive proinflammatory cytokines and autoantibodies, respectively (Lee et al., 2009). Indeed, it is reported that proinflammatory cytokines, such as tumor necrosis factor alpha (TNFα) or interferon-gamma (IFNγ), are elevated in the affected glands of SjS (Fox et al., 1994; Kolkowski et al., 1999). Moreover, those proinflammatory cytokines can induce apoptosis of salivary gland cells via caspase3 signaling (Kamachi et al., 2002; Kulkarni et al., 2006). On the other hand, it is thought that hypofunction of fluid secretion from affected glands is caused by autoantibody against muscarinic type 3 receptor (M3R) (Koo et al., 2008; Li et al., 2004). M3R is the major muscarinic acetylcholine (ACh) receptor in the salivary glands, and it plays a pivotal role in the induction of salivary fluid secretion (Baum, 1993). More specifically, ACh released from the parasympathetic nerves activates M3R to induce intracellular Ca^{2+} influx ($[Ca^{2+}]_i$) which initiates the cell signaling

required for fluid secretion from the acinar cells (Ambudkar, 2000; Ambudkar et al., 1993; Park et al., 2001). It is true that chemical agonists for M3R, such as pilocarpine and cevimeline, are often used clinically to stimulate the saliva secretion in SjS patients (Mavragani and Moutsopoulos, 2007).

M3R, a G-protein-coupled receptor (GPCR), belongs to the largest transmembrane receptor super-family in human and mice, and it is characterized by a seven transmembrane α-helix structure (Pierce et al., 2002). In general, the binding of an agonistic ligand to its cognate GPCR elicits Ca^{2+} and/or PKC signaling cascades that induce the expression of genes required for multiple fundamental functions, including exocrine and endocrine secretion, smooth muscle and cardiac contraction, pain transmission, fluid homeostasis, blood pressure, and immune response (Pierce et al., 2002). GPCRs, such as endothelin receptors and protease activated receptor I, also activate mitogenic signaling networks, such as PKC-PKD, MEK-ERK, or PI3K-Akt cascade, which lead to the induction of a variety of biological responses, including cell proliferation, differentiation, migration and survival (Rozengurt, 1998; Rozengurt, 2007).

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Many studies have shown the relevance of Ca^{2+} signaling in M3R-induced fluid secretion. However, such mitogenic signaling pathways as PKC, ERK and Akt, as well as their M3R-mediated downstream events, are still poorly understood in the context of salivary gland cells. Only recently has a muscarinic receptor agonist, carbachol (CCh), been reported to stimulate *in vitro* ERK phosphorylation in human immortalized salivary gland cells or rat submandibular acinar cells (Soltoff and Hedden, 2010). Since SjS is a chronic degenerative disease characterized by the gradual progression of cell apoptosis and resulting tissue destruction, it is plausible that mitogenic signaling, which can downregulate apoptosis induced by the caspase pathway (Steelman et al., 2008), could play a role in sustaining the configuration and functions of affected glands. Although it has been shown that M3R promotes neuroblastoma cell survival through activation of the ERK signaling pathway (Greenwood and Dragunow, 2010), it remains unclear whether M3R signaling in salivary gland cells is similarly cytoprotective against proinflammatory cytokine-induced apoptosis.

In the present study, we hypothesized that chemical agonist-mediated M3R activation can induce cell survival signaling in salivary gland cells, thereby contributing to the protection of cells against apoptosis caused by inflammatory insult. To test this

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hypothesis, apoptosis was induced in human salivary gland (HSG) cells by inflammatory stimulation with TNFα/IFNγ. CCh was employed as a chemical agonist for M3R based on studies showing that CCh can efficiently activate M3R expressed on cultured HSG cells (Cha et al., 2006; Pauley et al., 2011). We then investigated the cytoprotective effect of CCh-mediated M3R-ligation, as well as the molecular mechanism supporting such M3R-mediated cytoprotective effects.

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Materials and Methods

Chemicals

2-[(aminocarbonyl)oxy]-N,N,N-trimethylethanaminium chloride (Carbachol; CCh) was purchased from (Sigma, St. Louis, MO). Following chemicals were also employed as inhibitors for cell signaling molecules; 1,4-Diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene (U0126; Cell Signaling, Beverly, MA), 2-morpholin-4-yl-8-phenylchromen-4-one (LY294002; Cell Signaling), 4-Diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP; Tocris Bioscience, Ellisville, MO), 1,2-bis-(o-Aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid, tetraacetoxymethyl ester (BAPTA-AM; Tocris Bioscience), N-(3-chlorophenyl)-6,7-dimethoxy-4-quinazolinamine (AG1478; Cayman Chemical, Ann Arbor, MI), (4-amino-5-(4-chlorophenyl)-7-(dimethylethyl)pyrazolo[3,4-d]pyrimidine (PP2; ENZO Life Science, Plymouth Meeting, PA), and 2-[1-(3-Dimethylaminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)maleimide (GF109203X; ENZO Life Science).

Cell culture

Human salivary gland (HSG) cells, established from a human salivary gland (Sato et al.,

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1985), were used in this study. Cells were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10 % fetal bovine serum (FBS) (Hyclone Laboratories, Logan, Utah), penicillin G solution (100 units/ml), and streptomycin (100 μg/ml) (medium A) and maintained in a humidified atmosphere of 5 % CO_2 at 37 °C. The HSG cells were seeded at a density of 2.0×10^4 cells per well in 96-well plastic culture plates or 1.0×10^5 cells per well in 6-well plastic culture plates. Prior to the addition of any stimulants, the medium was changed to DMEM-supplemented antibiotics without FBS (medium B) and incubated for 4 hr. Then the cells were pretreated with or without U0126 (2.5 μM,), LY294002 (2.5 μM), 4-DAMP (1 μM,), BAPTA-AM (10 μM), AG1478 (5 μM), PP2 (5 μM), or non-toxic variant of diphtheria toxin protein (CRM197, 10 μM, Sigma) for 30 min in medium B. After such pretreatment, the HSG cells were exposed to 100 μM of CCh or 100 ng/ml of recombinant EGF (Peprotech, Rocky Hill, NJ) in the absence or presence of a TNFα (50 ng/ml, Peprotech)/IFNγ (10 ng/ml, Peprotech) combination for various periods. For chemical reagents dissolved in DMSO, the appropriate concentration of DMSO was added as solvent control.

Cell survival assay

The viable cells in each culture were enumerated with MTS assay by using Cell Titer

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96® Aqueous Non-Radioactive Proliferation Assay kit (Promega, Madison, WI) according to the manufacturer's instructions. In addition, TUNEL staining for apoptotic cells was performed using the DeadEnd™ Fluorometric TUNEL System (Promega). The fluorescence signals were detected with an Olympus FSX100 fluorescence microscopy (Olympus, Tokyo, Japan).

Measurement of caspase 3/7 activity

Caspase 3/7 activity was measured with a Caspase-Glo® Assay kit (Promega) according to the manufacturer's instructions. Luminescent intensity was monitored by a fluorometric imaging plate reader (Synergy HT, Biotech Instruments, Winooski, VT).

Immunoblotting

Cells were lysed in buffer containing 25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 0.1 % SDS, 1% NP-40, 10 % Glycerol, and 1% (v/v) Triton X-100 (Kajiya et al., 2011). The cell lysates were subjected to ultrasonic treatment for 8 sec on ice. The proteins in the cell lysates were separated using SDS/PAGE (12 % gel) and electrophoretically transferred onto a nitrocellulose (NC) membrane (Bio-Rad Laboratories, Hercules, CA). The NC membranes were

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blocked with 5 % skim milk for 1 hr, followed by the reaction with rabbit anti-human phosphorylated ERK antibody (Cell Signaling, 1:2000), rabbit anti-human total ERK antibody (Cell Signaling, 1:2000), rabbit anti-human phosphorylated Akt antibody (Cell Signaling, 1:2000), rabbit anti-human total Akt antibody (Cell Signaling, 1:200), rabbit anti-human phosphorylated PKCδ antibody (Cell Signaling, 1:500), rabbit anti-human total PKCδ antibody (Cell Signaling, 1:1000), rabbit anti-human phosphorylated EGFR antibody (Cell Signaling, 1:500), anti-human total EGFR antibody (Cell Signaling, 1:5000), rabbit anti-human phosphorylated Src family (Tyr416) antibody (Cell Signaling, 1:500), rabbit monoclonal anti-human total cellular Src (c-Src) antibody (clone: 36D10, Cell Signaling, 1:4000), and HRP-conjugated mouse anti-β-actin antibody (AbCam, Cambridge, MA; 1:5,000) at 4 °C overnight. After extensive washes, the NC membrane was incubated with peroxidase-conjugated donkey anti-rabbit IgG antibody (Jackson Immunoresearch, West Grove, PA; 1:5000) for 1 hr at room temperature. The localization of specific antibody that deposited to the molecule of interest on the NC membrane was detected by developing color using Immobilon Western Chemiluminescent HRP substrate (Millipore, Billerica, MA).

Immunoprecipitation

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Equal amounts of cell lysates (500 μg of total protein in 1 ml of the lysis buffer) were incubated with rabbit anti-human total EGFR antibody (1:50) at 4 ℃ overnight. Subsequently, GammaBind Plus Sepharose™ beads (Pharmacia Biotech, Uppsala, Sweden) were applied to the lysate/antibody mixture and incubated at 4 ℃ for 4 hr. After extensive washing with the lysis buffer, proteins captured by the anti-EGFR antibody-coated beads were separated in SDS-PAGE and subjected to Western blot analyses as described above. Especially, total c-Src protein level was determined by total anti-c-Src rabbit monoclonal antibody (clone: 32G6, Cell signaling, 1:500).

Intracellular Ca^{2+} Measurements

$[Ca^{2+}]_i$ was measured by using Fluo-8-no-wash calcium assay kit (AAT Bioquest, Sunnyvale, CA) as previously described (Ohta et al., 2011). Briefly, HSG cells were incubated in phenol red free Hank's buffer with 20 mM HEPES containing Fluo-8 NW dye-loading solution in the presence or absence of 4-DAMP (1 μM) or BAPTA-AM (10 μM) at 37 °C for 30 min. The cells were incubated at room temperature for another 30 min. Then, HSG cells were exposed to 100 μM of CCh for 1 min, and the fluorescence intensity (Ex/Em = 490/525 nm) was detected with a fluorometric imaging plate reader. The fluorescence intensities were quantified from three independent cell cultures.

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Transfection of siRNA

The validated PKCδ siRNA, EGFR siRNA or negative control siRNA were obtained from Invitrogen (ID numbers: #PRKCDVHS41574 for PKCδ siRNA, #EGFRVHS41680 for EGFR siRNA, and #12935-300 for negative control siRNA). HSG cells were seeded at a density of 1.25×10^4 cells per well in 24-well plastic culture plates in medium A and cultured at 37 °C for 24 hr. Then 20 nM of PKCδ siRNA, EGFR siRNA or negative control siRNA were transfected into the cells by using RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions. After 48 hr incubation, the cells treated with or without 100 μM of CCh were lysed, and immunoblotting assay was performed as described above.

Statistical analysis

Differences between two groups of interest were analyzed with Student's *t*-test.

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Results

CCh protects HSG cells from apoptosis induced by TNFα/IFNγ stimulation.

To examine the cytoprotective effect of M3R in HSG cells, muscarinic receptor agonist CCh was tested on TNFα/IFNγ-induced apoptosis, which was previously reported to activate apoptosis signaling in HSG cells (Kamachi et al., 2002; Kulkarni et al., 2006). Consistent with a previous report, the combined TNFα/IFNγ treatment reduced cell viability in HSG cells (Fig. 1A). However, CCh protected against cell death induced by TNFα/IFNγ in a dose-dependent manner (Fig. 1A). TUNEL staining showed that TNFα/IFNγ stimulation elevated the number of apoptotic cells, while CCh treatment significantly abrogated the elevation (Fig. 1B and 1C). Subsequently, we monitored the effect of CCh on the caspase3/7 death signal activity induced by TNFα/IFNγ challenge in HSG cells. As shown in Supplemental Figure 1A, caspase 3/7 activity reached a significantly higher level than control at 24 hr after stimulation with inflammatory cytokines. Therefore, in the following experiments, the measurements of caspase3/7 activity were performed 24 hr after TNFα/IFNγ challenge. It should be noted that the protocol for incubating HSG cells for 24 hours with proinflammatory cytokines to induce caspase activity was previously published by Kulkarni et al. (Kulkarni et al., 2006). The increased caspase3/7 activity induced by TNFα/IFNγ (Fig. 1D) was

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significantly attenuated by the addition of CCh (Fig. 1D). Interestingly, 4hr or longer pre-exposure of CCh did not show inhibitory effects of CCh on TNFα/IFNγ-induced caspase3/7 activity (Supplemental Fig. 1B). However, both simultaneous addition of CCh and 1hr pretreatment with CCh attenuated TNFα/IFNγ-induced caspase3/7 activity (Supplemental Figure 1B), indicating that CCh-mediated anti-apoptosis signaling is effective in the 0 - 1 hour range. Taken together, these findings suggested that CCh can protect HSG cells from TNFα/IFNγ-induced apoptotic signaling.

CCh activates ERK and Akt signaling cascades, which play a crucial role in protecting HSG cells from inflammatory cytokine-induced apoptosis.

To understand how CCh-induced signaling protects HSG cells from apoptotic challenge, we explored the influence of CCh on ERK and Akt phosphorylation, which initiates signaling for cell survival in many different types of cells (Chappell et al., 2011; Kajiya et al., 2009; Steelman et al., 2008). CCh treatment dramatically increased the phosphorylation of ERK and Akt, both peaking at 5 min and then gradually decreasing (Fig. 2A and 2B). In addition, while CCh-induced ERK phosphorylation was inhibited by U0126 (MEK-ERK1/2 inhibitor), LY294002 (PI3-Kinase-Akt inhibitor) had no effect (Figure 2C). On the other hand, the upregulation of Akt phosphorylation caused

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by CCh was remarkably inhibited by LY294002, but not by U0126 (Figure 2C). It is noteworthy that neither U0126 nor LY294002 affected TNFα/IFNγ-induced caspase3/7 activity, while those inhibitors clearly attenuated the CCh-mediated protective effect in HSG cells (Fig 2D and 2E). Interestingly, the stimulation of TNFα/IFNγ transiently increased phosphorylation of ERK and Akt, whereas such transit phosphorylation diminished to lower than basal level after 20 min from the challenge by inflammatory cytokines (Supplemental Fig. 2A and 2B), suggesting that the net impact of TNFα/IFNγ on ERK and Akt phosphorylation levels in HSG cells was suppressive. These findings indicated that the upregulation of both ERK and Akt signaling induced by CCh is responsible for its cytoprotective effect in HSG cells.

M3R-activation is responsible for CCh-mediated cell survival signaling in HSG cells

To examine the relationship between M3R-activation and CCh-induced cell survival signaling, M3R antagonist 4-DAMP was employed. The pretreatment of 4-DAMP abrogated the phosphorylation of both ERK and Akt induced by CCh in HSG cells (Fig. 3A). Furthermore, the CCh-mediated protective effect on TNFα/IFNγ-induced caspase3/7 activity was significantly inhibited by 4-DAMP. These findings suggested that CCh activates the ERK and Akt mitogenic survival signaling cascade via M3R in

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HSG cells.

Neither Ca^{2+} nor PKCδ signaling is associated with CCh-induced cytoprotective ERK and Akt signaling cascade

It is well known that Ca^{2+} signaling, which is caused by M3R activation, plays pivotal roles in fluid secretion of salivary gland cells (Koo et al., 2008; Li et al., 2004); therefore, we asked whether Ca^{2+} signaling, in addition to the activation of ERK and Akt signaling, as described above, could also be involved in CCh-induced cell survival signaling in HSG cells. As expected, CCh did upregulate $[Ca^{2+}]_i$, while both M3R inhibitor (4-DAMP) and Ca^{2+}-chelator (BAPTA-AM) suppressed CCh-induced increase in $[Ca^{2+}]_i$ in HSG cells (Fig. 4A). Interestingly, however, BAPTA-AM, which completely blocked calcium mobility in HSG cells, failed to inhibit CCh-induced phosphorylation of ERK and Akt (Fig. 4B). Moreover, CCh-mediated suppressive effect on TNFα/IFNγ-induced caspase3/7 activity was not affected by BAPTA-AM treatment (Fig. 4C). These findings indicated that CCh causes cell survival mitogenic signaling in a Ca^{2+}-independent manner.

Next, we investigated the possible engagement of PKCδ in M3R signaling because activation of PKC has been linked to muscarinic receptor-induced ERK activation in

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several cell types (Jimenez and Montiel, 2005; Keely et al., 1998). In addition, based on the following lines of evidence, we focused on PKCδ expressed in HSG cells. First, CCh causes cell survival mitogenic signaling in a $[Ca^{2+}]_i$-independent manner (Figure 4). Second, in contrast to the major isoforms of PKC (α, βI, βII and γ) that require $[Ca^{2+}]_i$ for their full activation, activation of the second class of PKC isoforms, including PKCδ, occurs in a fashion independent of $[Ca^{2+}]_i$ (Parker and Murray-Rust, 2004). Third, B lymphocytes infiltrating the salivary glands of SjS patients could cause epithelial cell apoptosis via activation of PKCδ (Varin et al., 2012). We found that CCh increased the phosphorylation level of PKCδ in a time-dependent manner (Fig. 4D) and that such CCh-induced PKCδ upregulation was blocked by 4-DAMP (Fig. 4E), indicating that PKCδ is activated via M3R in HSG cells. The transfection of PKCδ siRNA diminished PKCδ expression (Fig. 4F). However, contrary to our expectation, knockdown of PKCδ did not affect the CCh-induced phosphorylation of ERK and Akt in HSG cells (Fig. 4G), while phosphorylated PKCδ expression was undetectable (data not shown). Furthermore, PKCδ siRNA failed to attenuate CCh-mediated protective effect against TNFα/IFNγ-induced caspase3/7 activity in HSG cells (Fig. 4H). These findings demonstrated that CCh-induced cell survival signaling is independent of PKCδ signaling in HSG cells.

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EGFR transactivation is involved in CCh-induced cell survival signaling.

It is reported that some muscarinic receptors can induce ERK activation via transactivation of EGFR in various cell types (Kanno et al., 2003; Keely et al., 2000). Therefore, we tested whether CCh-induced cell survival signaling is mediated by EGFR transactivation. CCh increased the phosphorylation level of EGFR in a time-dependent manner (Fig. 5A), but such elevation of phospho-EGFR was blocked by the M3R inhibitor 4-DAMP (Fig. 5B), indicating that EGFR is transactivated via M3R in HSG cells. Subsequently, in order to test whether EGFR phosphorylation is involved in CCh-induced cell survival signaling, we next performed an inhibition assay using AG1478, an EGFR kinase-specific inhibitor. The pretreatment of HSG cells with AG1478 abrogated the CCh-induced phosphorylation of EGFR, ERK and Akt (Fig. 5C). Furthermore, the protective effect of CCh on TNFα/IFNγ-induced caspase3/7 activity was also significantly diminished by AG1478 (Fig. 5D). To confirm these findings, we also performed an RNAi-based gene silencing assay using EGFR siRNA. The transfection of EGFR siRNA decreased the EGFR expression level in HSG cells (Fig. 5E). Importantly, EGFR siRNA transfection attenuated the CCh-induced ERK and Akt phosphorylation (Fig. 5F). These findings suggested that M3R ligation with CCh leads

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to EGFR transactivation which, in turn, initiates the cytoprotective survival signaling in HSG cells.

Intracellular signal adaptor, c-Src, intervenes between M3R and EGFR in the CCh-induced EGFR transactivation cascade.

It is known that GPCR (such as muscarinic receptors)-induced EGFR transactivation is dependent on the activation of cell signal adaptor c-Src, a non-receptor tyrosine kinase, in various cell types (Rosenblum et al., 2000; Yeh et al., 2005). Therefore, we asked whether c-Src could be associated with CCh-induced transactivation of EGFR signaling in HSG cells. CCh caused the rapid transient phosphorylation of Src at Tyr416, indicating the active state of the kinase, which began to express as early as 1 min following stimulation with CCh (Fig. 6A). Importantly, the immunoprecipitation assay also showed that CCh stimulation enhanced the association between c-Src and EGFR in a time-dependent manner, which was detectable after 1 min (Fig. 6B). Moreover, 4-DAMP treatment blocked both CCh-induced phosphorylation of c-Src at Tyr416 (Fig. 6C) and the association of c-Src with EGFR (Fig. 6D). It is noteworthy that Src tyrosine kinase inhibitor PP2 reduced CCh-induced phosphorylation of EGFR, ERK and Akt in HSG cells (Fig. 6E). Finally, the protective effect of CCh against TNFα/IFNγ-induced

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caspase3/7 activity was also significantly attenuated by PP2 (Fig. 6F). Taken together, these findings demonstrated that CCh-induced Src activation via M3R causes EGFR transactivation, leading to cell survival signaling mediated by ERK and Akt phosphorylation in HSG cells.

Exogenously applied EGF emulates CCh-induced cytoprotective cell signaling

If it is true that transactivation of EGFR via M3R can induce survival mitogenic signaling, then direct, i.e., exogenous, activation of EGFR should induce mitogenic survival signaling in HSG cells. To test this supposition, we examined the effect of exogenously applied EGF on 1) EGFR, ERK and Akt phosphorylation and 2) TNFα/IFNγ-induced caspase3/7 activity in HSG cells. Exogenously applied EGFR increased the phosphorylation level of EGFR (Fig. 7A), ERK (Fig. 7B) and Akt (Fig. 7C) in a time-dependent manner. Most importantly, EGF treatment attenuated TNFα/IFNγ-induced caspase3/7 activity (Fig. 7D). Moreover, it is noteworthy that both MEK-ERK inhibitor U0126 and PI3Kinase-Akt inhibitor LY294002 significantly diminished the cytoprotective effects of EGF (Fig. 7D). These findings clearly suggested that EGFR activation plays a critical role in the protection of salivary gland cells from apoptotic challenge.

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Discussion

For the first time, the present study revealed that ligation of M3R, via transactivation of EGFR, can upregulate not only ERK, but also Akt, which, in turn, suppresses the caspase3/7 death signal, as well as apoptosis of HSG cells induced by inflammatory insult (Fig. 8). Although Akt and ERK signaling are major intracellular signaling pathways for cell survival (Chappell et al., 2011; Kajiya et al., 2009; Steelman et al., 2008), many previous studies reporting on the pathophysiology of HSG cells were focused on understanding M3R-induced Ca^{2+} signaling in the fluid secretion system (Koo et al., 2008; Li et al., 2004; Tobin et al., 2009), not the underlying cytoprotective mechanism. Soltoff et al. have demonstrated that activation of M3R promotes ERK phosphorylation in salivary gland cells (Soltoff and Hedden, 2010), supporting our finding of CCh-mediated activation of ERK and Akt phosphorylation. However, the finding in the present study, i.e., that activation of M3R elicits cytoprotective signals via transactivation of EGFR, provides a novel scientific foundation for understanding the pathophysiology of SjS and offers new molecular targets for SjS drug discovery.

The fact that some GPCR agonists can transactivate EGFR was originally reported in 1996 (Daub et al., 1996), and this theory is now applied to a wide variety of GPCR

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ligands (Gschwind et al., 2001). This paradigm requires the intervention of the intracellular signal adaptor molecule c-Src which activates the intracellular tyrosine kinase domain of EGFR (Amorino et al., 2007; Rozengurt, 2007), as shown in this study (Fig. 6). However, recent studies demonstrated that transactivation of EGFR by GPCR ligands can occur through both intracellular c-Src intervention and extracellular EGFR-ligand transfer (Bhola and Grandis, 2008; Ohtsu et al., 2006). More specifically, activation of GPCR induces the expression of extracellular enzymes, which cleave the ectodomain of heparin binding (HB)-EGF (cell membrane-bound form of EGFR ligands). Subsequently, the released (HB)-EGF binds and activates EGFR (Bhola and Grandis, 2008; Ohtsu et al., 2006). To test if such alternative mechanism of extracellular M3R-EGFR transactivation is involved in CCh-stimulated HSG cells, we explored the effect of HB-EGF inhibitor CRM197 on CCh-induced EGFR, ERK and Akt phosphorylation (Supplemental Fig. 3). However, since the HB-EGF inhibitor neither blocked CCh-induced EGFR transactivation nor ERK/Akt phosphorylation (Supplemental Fig. 3), M3R-induced EGFR transactivation in HSG cells appeared to be solely regulated by intracellular c-Src intervention between M3R and EGFR.

In addition to CCh-induced EGFR transactivation that involved intervention of Src

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activation, the present study demonstrated that EGFR activation induced by exogenously applied EGF is involved in the cytoprotective signaling in HSG cells. However, although exogenously applied EGF elicited full inhibition of caspase3/7 activity induced by TNFα/IFNγ (Fig. 7D), it is noteworthy that the CCh-mediated suppression of caspase3/7 activity was rather modest (about 50-60% inhibition) (Fig .2D and 2E). In addition, CCh induced differences in both time course and signal intensity of EGFR, ERK, and Akt phosphorylation (Fig. 2A,2B and 5A) compared to that induced by EGF (Fig. 7A-7C). More specifically, EGF induced EGFR phosphorylation and mitogenic signaling activity more rapidly and dramatically than CCh. These findings indicated that the intensity of CCh-evoked EGFR transactivation was attenuated by the intervention of M3R-Src signaling process compared to direct activation of EGFR with EGF.

Ligation of M3R with its cognate agonist induces classical Gq protein-mediated PLCβ activation, leading to the production of the second messengers inositol trisphosphate and diacylglycerol, which causes the elevation of $[Ca^{2+}]_i$ as well as activation of PKCs (Singer et al., 1997). In accordance with this classical theory, CCh stimulated intracellular Ca^{2+} mobilization and PKCδ phosphorylation via M3R in HSG cells (Fig.

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4). However, even though these cell signaling cascades have been demonstrated to regulate saliva formation and secretion, CCh mediation of these classical pathways was not associated with cell survival signaling in HSG cells (Fig. 4) (Ambudkar et al., 1993; Soltoff et al., 1998; Soltoff and Toker, 1995).

Based on fact that the PKC family is composed of more than 15 isozymes, in addition to PKCδ siRNA, we have also tested the pan-PKC inhibitor GF109203X on CCh-induced cytoprotective signaling in HSG cells. Consistent with the results from PKCδ siRNA experiments (Fig. 4), the PKC inhibitor failed to attenuate CCh-induced ERK and Akt phosphorylation or its cytoprotective effect against TNFα/IFNγ-induced caspase3/7 activation (Supplemental Fig. 4A and 4B). Nevertheless, it is still possible that other PKCs are associated with the CCh-induced mitogenic signaling, essentially because GF109203X inhibits only PKCα, PKCβI, PKCδ, and PKCε, but it has little or no effect on other PKC isoforms, such as PKCβII and PKCγ. The total absence of PKCβII in the acinar epithelial cells of patients with SjS has been reported (Tornwall et al., 1997), implicating that PKCβII activation may not be associated with inflammation-caused caspase3/7 activation in the context of SjS lesion. However, it is still conceivable that other GF109203X-resistant PKC isoforms, such as PKCγ, may participate in

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CCh-mediated cytoprotective signaling. For example, when effects of EGFR inhibitor (AG1478: Fig. 5C) on the CCh-elicited phosphorylation of EGFR, ERK and Akt were compared to those of Src inhibitor (PP2: Fig. 6E), discrepancies in the suppression levels of phosphorylation by these two drugs were evident, suggesting the possible intervention of other signaling pathways between Src and EGFR. Consequently, additional comprehensive study will be required to elucidate the detailed signaling networks that are engaged in CCh-induced cytoprotective events.

Lin et al. recently reported that 1) CCh stimulates ERK phosphorylation via PKC activity without EGFR transactivation, whereas 2) another muscarinic agonist, pilocarpine, could upregulate ERK phosphorylation through c-Src-dependent EGFR transactivation in human salivary gland cell line HSY cells (Lin et al., 2008). Unlike the present study that addressed the effects of CCh on cytoprotection, Lin et al. only focused on CCh-induced ERK signaling without considering the downstream outcomes of ERK activation. Most importantly, HSG cells usedin the present study predominantly express M3R among all muscarinic receptor isoforms (Nagy et al., 2007), while HSY cells express both M1 and M3 receptors to an equal degree (Lin et al., 2008). To explain the discrepancy in the findings of CCh's actions between the study by Lin et

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al. and the present study, it is assumed that CCh binding to M1R in HSY cells may have induced ERK phosphorylation without EGFR transactivation, while CCh-mediated M3R activation resulted in EGFR transactivation-dependent ERK activation in the present study. Nonetheless, the study by Lin et al. still supports our key finding, i.e., that activation of M3R can lead to cytoprotective ERK activation signal via EGFR transactivation in HSG cells.

In the native salivary gland tissue, CCh-induced elevations of cytoplasmic free calcium concentrations ($[Ca^{2+}]i$) stimulate fluid secretion in salivary acinar cells via activating apically-located Cl^- channels and basolaterally-located K^+ channels (Romanenko, et al., 2006). In this context, CCh appears to increase the turnover of Na-K ATPase, while reducing intracellular ATP, which, in turn, promotes the activation of AMP-activated protein kinase (AMPK)(Soltoff, 2004). Very interestingly, it is reported that adiponectin can prevent IFN-g-induced apoptosis of salivary gland cells through AMPK activation (Katsiougiannis et al., 2010), suggesting that the signaling that can activate AMPK, including CCh-evoked M3R stimulation, may also protect salivary gland cell apoptosis. On the other hand, immunohistochemical analysis of diseased gland tissue of SjS patient demonstrated that surviving salivary gland cells strongly showed phosphorylated

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EGFR expression, even though the number of apoptotic cells was increased (Nakamura et al., 2007). Dang et al. also reported that salivary gland injury induced in rats upregulates EGFR phosphorylation in salivary acinar cells, which, in turn, protects cells against apoptosis (Dang et al., 2008). These latter two reports indicate that EGFR of salivary gland cells is activated in the physiological context of SjS lesion. Finally, the fact anti-EGFR monoclonal antibodies, such as cetuximab, can suppress recurrence and/or metastasis of salivary gland carcinoma (Locati et al., 2009) strongly supports the finding that EGFR-evoked signaling is engaged in salivary gland cell survival in the native tissue. Therefore, the application of EGF or ligation of receptors, including M3R, that can trigger the transactivation of EGFR, may constitute a novel therapeutic regimen to ameliorate the gland destruction observed in SjS patients.

Interestingly, while M3R chemical agonists, such as pilocarpine and cevimeline, are often used clinically to stimulate salivary secretion in SjS patients (Mavragani and Moutsopoulos, 2007), no studies have ever addressed whether such treatment can also protect against the progression of grand destruction. However, based on our results, it is plausible that clinical treatment using M3R chemical agonists could offer cytoprotective effects against proinflammatory cytokine-induced apoptosis in salivary

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gland cells, particularly in the context of tissue destruction that occurs in SjS patients.

In summary, CCh-stimulated M3R transactivates EGFR via a signal intervention mediated by c-Src, which results in phosphorylation of both ERK and Akt. As a consequence, the culmination of these signaling events attenuates TNFα/IFNγ-induced caspase3/7 activity and protects the salivary gland cells from apoptosis (Fig. 8). Therefore, this study sheds light on the EGFR transactivation system and introduces novel molecular targets to the search for a therapeutic chemical compound that can protect HSG cells from inflammation-induced apoptosis, potentially leading to the development of novel therapeutic interventions against SjS.

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Acknowledgement

This study was supported, in part, by NIH RO1 grant DE019644 from the NIDCR.

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Authorship Contribution

Participated in research design: Kajiya, Jin, Yu, Cha, Kawai

Conducted experiments: Kajiya, Ichimonji, Min, Zhu, Almazrooa

Contributed new reagents or analytic tools: Kajiya, Cha

Performed data analysis: Kajiya, Ichimonji, Min, Zhu, Almazrooa

Wrote or contributed to the writing of the manuscript: Kajiya, Jin, Yu, Cha, Kawai

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Footnotes

This study was supported, in part, by National Institute of Dental and Craniofacial Research [DE019644].

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Fig. 1. CCh protects HSG cells from proinflammatory cytokine-induced apoptosis.

(A) HSG cells were treated with or without various concentrations of CCh (10-1000 μM) in the absence or presence of combined TNFα (50 ng/ml)/IFNγ (10 ng/ml) and incubated for 56 hr. Cell viability was determined as described in the Materials and Methods section and is shown as a percentage of the viability of the cells that were grown in medium B. Values represent means ± S.D. of four cultures. * $p < 0.05$, ** $p < 0.01$: Values differ significantly (*t*-test). (B-D) HSG cells were treated with or without 100 μM of CCh in the presence or absence of combined TNFα (50 ng/ml)/IFNγ (10 ng/ml) and incubated for 40 hr (B and C) or 24 hr (D). TUNEL-positive apoptotic cells (green) are shown under each set of conditions (B), and the graph shows the percentage of TUNEL-positive apoptotic cells (C). Values represent means ± S.D. of three cultures. ** $p < 0.01$: Value differs significantly (*t*-test). Similar results were obtained from three experiments. (D) Caspase3/7 activity was indicated by luminescence activity as described in the Materials and Methods section. Values represent means ± S.D. of three cultures. * $p < 0.05$, ** $p < 0.01$: Values differ significantly (*t*-test).

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Fig. 2. ERK and Akt signaling pathways are essential for CCh-promoted cytoprotection against apoptotic challenge in HSG cells.

(A and B) HSG cells were exposed to CCh (100 μM) for the indicated times. The phosphorylated or total ERK (A) and Akt (B) levels were analyzed by immunoblotting. (C) HSG cells were pretreated with or without U0126 (2.5 μM) or LY294002 (2.5 μM) for 30 min and then exposed to CCh (100 μM) for 10 min. The phosphorylated or total ERK and Akt levels were analyzed by immunoblotting. Quantification of the band density was performed by densitometric scanning of each band using NIH image software. (D and E) HSG cells were pretreated with or without U0126 (2.5 μM) or LY294002 (2.5 μM) for 30 min. Then the cells were treated with or without 100 μM of CCh in the presence or absence of combined TNFα (50 ng/ml)/IFNγ (10 ng/ml) and incubated for 24 hr. Caspase3/7 activity was indicated by luminescence activity as described in the Materials and Methods section. Values represent means ± S.D. of three cultures. * $p < 0.05$, ** $p < 0.01$: Values differ significantly (*t*-test).

Fig. 3. M3R is involved in CCh-induced cell survival signaling in HSG cells.

(A) HSG cells were pretreated with or without 4-DAMP (1 μM) for 30 min and then exposed to CCh (100 μM) for 10 min. The phosphorylated or total ERK and Akt levels

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were analyzed by immunoblotting. Quantification of the band density was performed by densitometric scanning of each band using NIH ImageJ software. (B) HSG cells were pretreated with or without 4-DAMP (1 μM) for 30 min. Then the cells were treated with or without 100 μM of CCh in the presence or absence of combined TNFα (50 ng/ml)/IFNγ (10 ng/ml) and incubated for 24 hr. Caspase3/7 activity was indicated by luminescence activity as described in the Materials and Methods section. Values represent means ± S.D. of three cultures. * $p < 0.05$, ** $p < 0.01$: Values differ significantly (*t*-test).

Fig. 4. Neither Ca^{2+} nor PKCδ signaling is associated with CCh-induced cytoprotective ERK and Akt signaling cascade.

(A) HSG cells were pretreated with or without 4-DAMP (1 μM) or BAPTA-AM (10 μM) for 30 min and then exposed to CCh (100 μM), followed by immediate measurement of 8-Fluo fluorescent intensity to monitor intracellular calcium mobilization. Relative fluorescence intensity ratios are presented as the mean ± SD of three independent experiments relative to that of medium alone. ** $p < 0.01$: Values differ significantly (*t*-test) ND: No significant difference. (B) HSG cells were pretreated with or without BAPTA-AM (10 μM) for 30 min and then exposed to CCh (100 μM)

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for 10 min. The phosphorylated or total ERK and Akt levels were analyzed by immunoblotting. (C) HSG cells were pretreated with or without BAPTA-AM (10 μM) for 30 min. Then the cells were treated with or without 100 μM of CCh in the presence or absence of combined TNFα (50 ng/ml)/IFNγ (10 ng/ml) and incubated for 24 hr. Caspase3/7 activity was indicated by luminescence activity. Values represent means ± S.D. of three cultures. ** $p < 0.01$: Values differ significantly (*t*-test). (D) HSG cells were exposed to CCh (100 μM) for the indicated times. The phosphorylated or total PKCδ levels were analyzed by immunoblotting. (E) HSG cells were pretreated with or without 4-DAMP (1 μM) for 30 min and then exposed to CCh (100 μM) for 5 min. The phosphorylated or total PKCδ levels were analyzed by immunoblotting. (F) HSG cells, having been transfected with negative control (neg) or PKCδ siRNA, were cultured for 48 hr in medium A. The levels of PKCδ and β-actin in the cells were analyzed by immunoblotting. (G) HSG cells, having been transfected with negative control (neg) or PKCδ siRNA, were cultured for 48 hr in medium A and were then exposed to CCh (100 μM) for 5 min in medium B. The phosphorylated or total ERK, Akt and PKCδ levels were analyzed by immunoblotting. Quantification of the band density was performed by densitometric scanning of each band using NIH ImageJ software. (H) HSG cells, having been transfected with negative control (neg) or PKCδ siRNA, were

cultured for 48 hr in medium A and were pretreated with or without BAPTA-AM (10 μM) for 30 min. Then the cells were treated with or without 100 μM of CCh in the presence or absence of combined TNFα (50 ng/ml)/IFNγ (10 ng/ml) and incubated for 24 hr. Caspase3/7 activity was indicated by luminescence activity. Values represent means ± S.D. of three cultures. * $p < 0.05$, ** $p < 0.01$: Values differ significantly (*t*-test).

Fig. 5. EGFR transactivation is involved in CCh-induced cell survival signaling.

(A) HSG cells were exposed to CCh (100 μM) for the indicated times. The phosphorylated or total EGFR levels were analyzed by immunoblotting. (B and C) HSG cells were pretreated with or without 1 μM of 4-DAMP (B) or 5 μM of AG1478 (C) for 30 min and then exposed to CCh (100 μM) for 5 min. The phosphorylated or total EGFR, ERK, and Akt levels were analyzed by immunoblotting. (D) HSG cells were pretreated with or without AG1478 (5 μM) for 30 min. Then the cells were treated with or without 100 μM of CCh in the presence or absence of combined TNFα (50 ng/ml)/IFNγ (10 ng/ml) and incubated for 24 hr. Caspase3/7 activity was indicated by luminescence activity as described in the Materials and Methods section. Values represent means ± S.D. of three cultures. ** $p < 0.01$: Value differs significantly (*t*-test).

(E) HSG cells, having been transfected with negative control (neg) or EGFR siRNA, were cultured for 48 hr in medium A. The levels of EGFR and β-actin in the cells were analyzed by immunoblotting. (F) HSG cells, having been transfected with negative control (neg) or EGFR siRNA, were cultured for 48 hr in medium A and were then exposed to CCh (100 μM) for 5 min in medium B. The phosphorylated or total ERK, Akt and EGFR levels were analyzed by immunoblotting. Quantification of the band density was performed by densitometric scanning of each band using NIH ImageJ software.

Fig. 6. Src activation is essential for CCh-induced EGFR transactivation cascade, resulting in cytoprotection against apoptotic challenge.

(A and B) HSG cells were exposed to CCh (100 μM) for the indicated times. (C and D) HSG cells were pretreated with or without 4-DAMP (1 μM) for 30 min and then exposed to CCh (100 μM) for 2 min. The phosphorylated or total Src levels were analyzed by immunoblotting (A and C). Cell lysates were prepared and used for immunoprecipitation (IP) with total EGFR antibody. The levels of c-Src protein co-immunoprecipitated with EGFR or total amount of EGFR were visualized by immunoblotting (IB) (B and D). (E) HSG cells were pretreated with or without PP2 (5

Molecular Pharmacology Fast Forward. Published on April 17, 2012 as DOI: 10.1124/mol.111.077354
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μM) for 30 min and then exposed to CCh (100 μM) for 2 min. The phosphorylated or total EGFR, ERK, and Akt levels were analyzed by immunoblotting. Quantification of the band density was performed by densitometric scanning of each band using NIH ImageJ software. (F) HSG cells were pretreated with or without PP2 (5 μM) for 30 min. Then the cells were treated with or without 100 μM of CCh in the presence or absence of combined TNFα (50 ng/ml)/IFNγ (10 ng/ml) and incubated for 24 hr. Caspase3/7 activity was indicated by luminescence activity as described in the Materials and Methods section. Values represent means ± S.D. of three cultures. ** $p < 0.01$: Value differs significantly (*t*-test).

Fig. 7. Exogenously applied EGF activates both ERK and Akt signaling cascade, which results in inhibition of Caspase3/7 activity induced by inflammatory apoptotic challenge in HSG cells.

(A-C) HSG cells were exposed to exogenously applied EGF (100 ng/ml) for the indicated times. The phosphorylated or total EGFR (A), ERK (B) and Akt (C) levels were analyzed by immunoblotting. The phosphorylated or total EGFR, ERK and Akt levels were analyzed by immunoblotting. Quantification of the band density was performed by densitometric scanning of each band using NIH ImageJ software. (D)

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HSG cells were pretreated with or without U0126 (2.5 μM) or LY294002 (2.5 μM) for 30 min. Then the cells were treated with or without 100 ng/ml of EGF in the presence or absence of combined TNFα (50 ng/ml)/IFNγ (10 ng/ml) and incubated for 24 hr. Caspase3/7 activity was indicated by luminescence activity as described in the Materials and Methods section. Values represent means ± S.D. of three cultures. * $p < 0.05$, ** $p < 0.01$: Values differ significantly (*t*-test).

Fig. 8. Schematic summary of CCh-induced M3R signaling cascade in HSG cells.

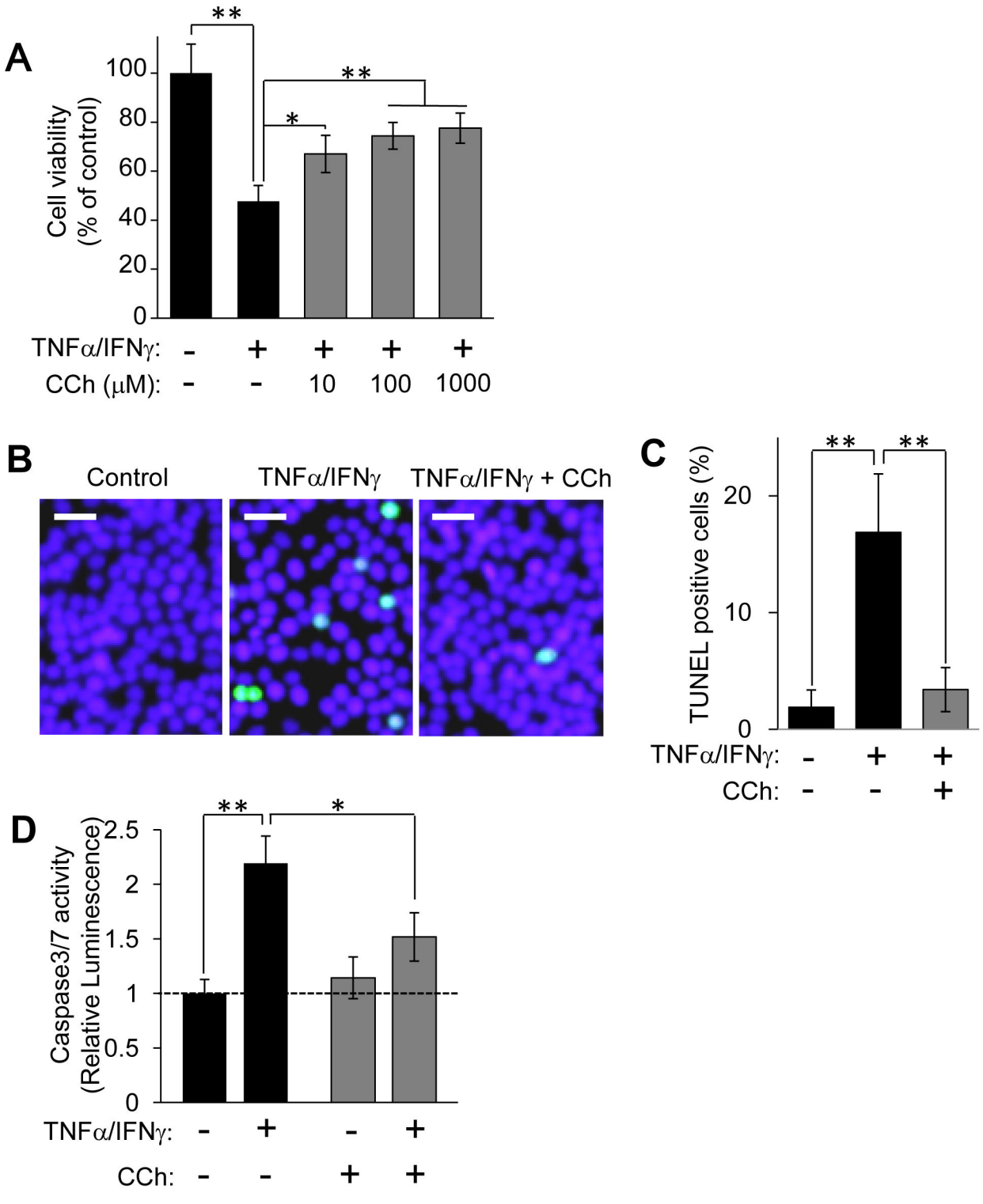

A
Cell viability (% of control)
100
80
60
40
20
0
**
**
*
TNFα/IFNγ: - + + + +
CCh (μM): - - 10 100 1000
B
Control
TNFα/IFNγ
TNFα/IFNγ + CCh
C
TUNEL positive cells (%)
20
10
0
**
**
TNFα/IFNγ: - + +
CCh: - - +
D
Caspase3/7 activity (Relative Luminescence)
2.5
2
1.5
1
0.5
0
**
*
TNFα/IFNγ: - + - +
CCh: - - + +

Figure 1

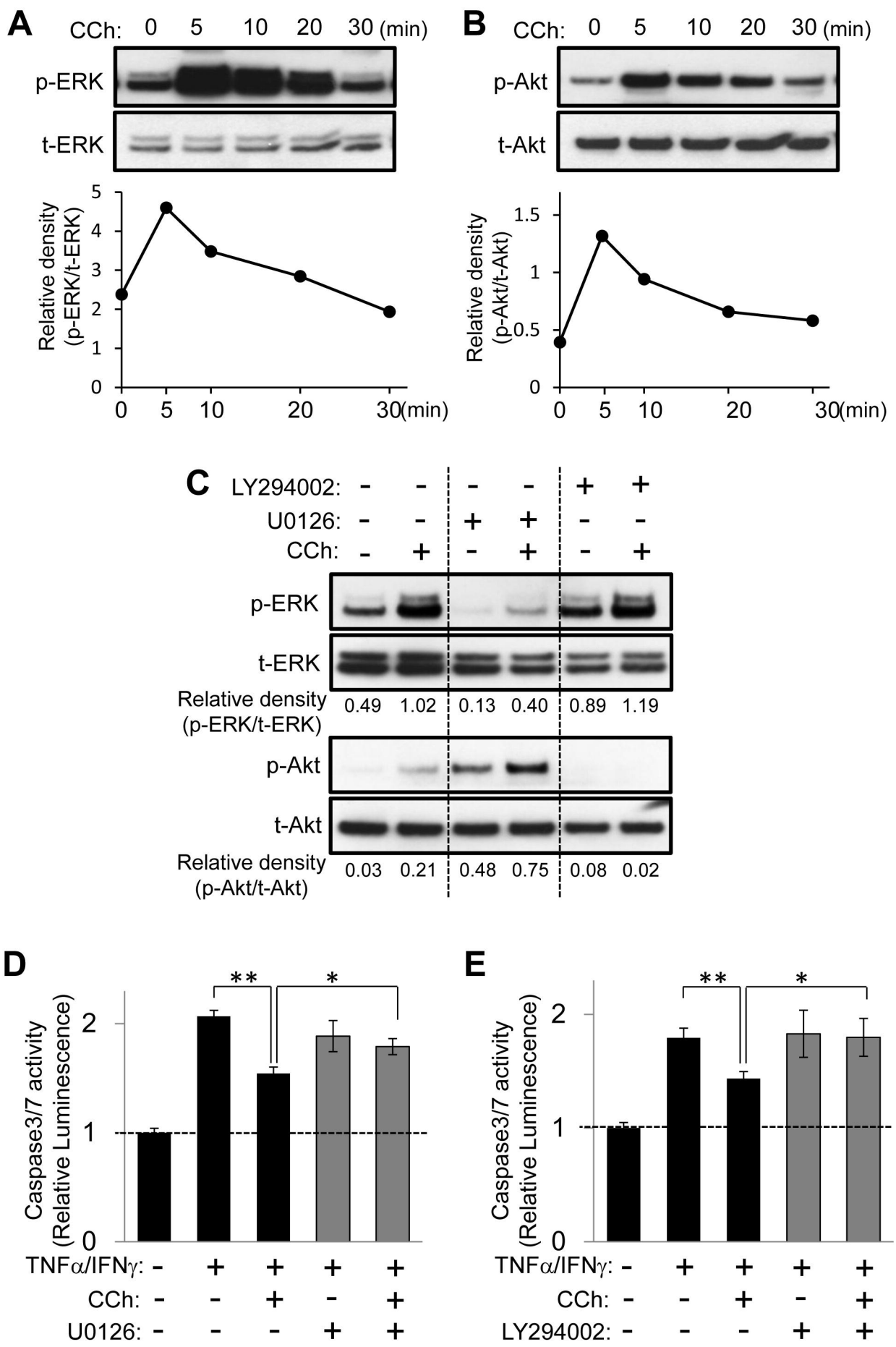

A
CCh: 0 5 10 20 30 (min)
p-ERK
t-ERK
Relative density (p-ERK/t-ERK)
0 5 10 20 30(min)
B
CCh: 0 5 10 20 30 (min)
p-Akt
t-Akt
Relative density (p-Akt/t-Akt)
0 5 10 20 30(min)
C
LY294002: - - - - + +
U0126: - - + + - -
CCh: - + - + - +
p-ERK
t-ERK
Relative density (p-ERK/t-ERK)
0.49 1.02 0.13 0.40 0.89 1.19
p-Akt
t-Akt
Relative density (p-Akt/t-Akt)
0.03 0.21 0.48 0.75 0.08 0.02
D
Caspase3/7 activity (Relative Luminescence)
**
*
TNFα/IFNγ: - + + + +
CCh: - - + - +
U0126: - - - + +
E
Caspase3/7 activity (Relative Luminescence)
**
*
TNFα/IFNγ: - + + + +
CCh: - - + - +
LY294002: - - - + +

Figure 2

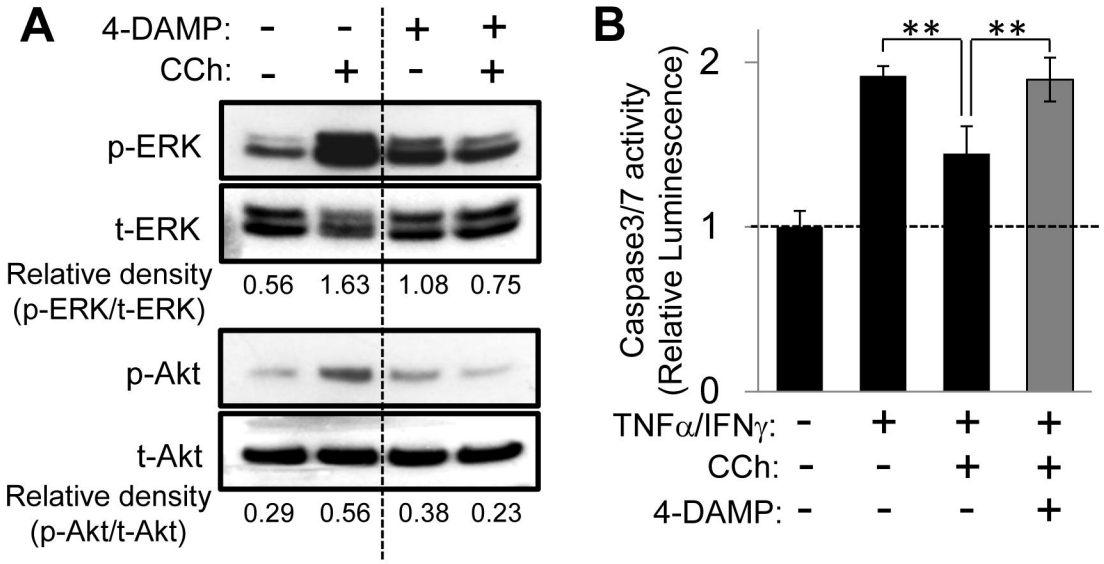

A
4-DAMP: - - + +
CCh: - + - +
p-ERK
t-ERK
Relative density
(p-ERK/t-ERK)
0.56 1.63 1.08 0.75
p-Akt
t-Akt
Relative density
(p-Akt/t-Akt)
0.29 0.56 0.38 0.23
B
Caspase3/7 activity
(Relative Luminescence)
2
1
0
**
**
TNFα/IFNγ: - + + +
CCh: - - + +
4-DAMP: - - - +

Figure 3

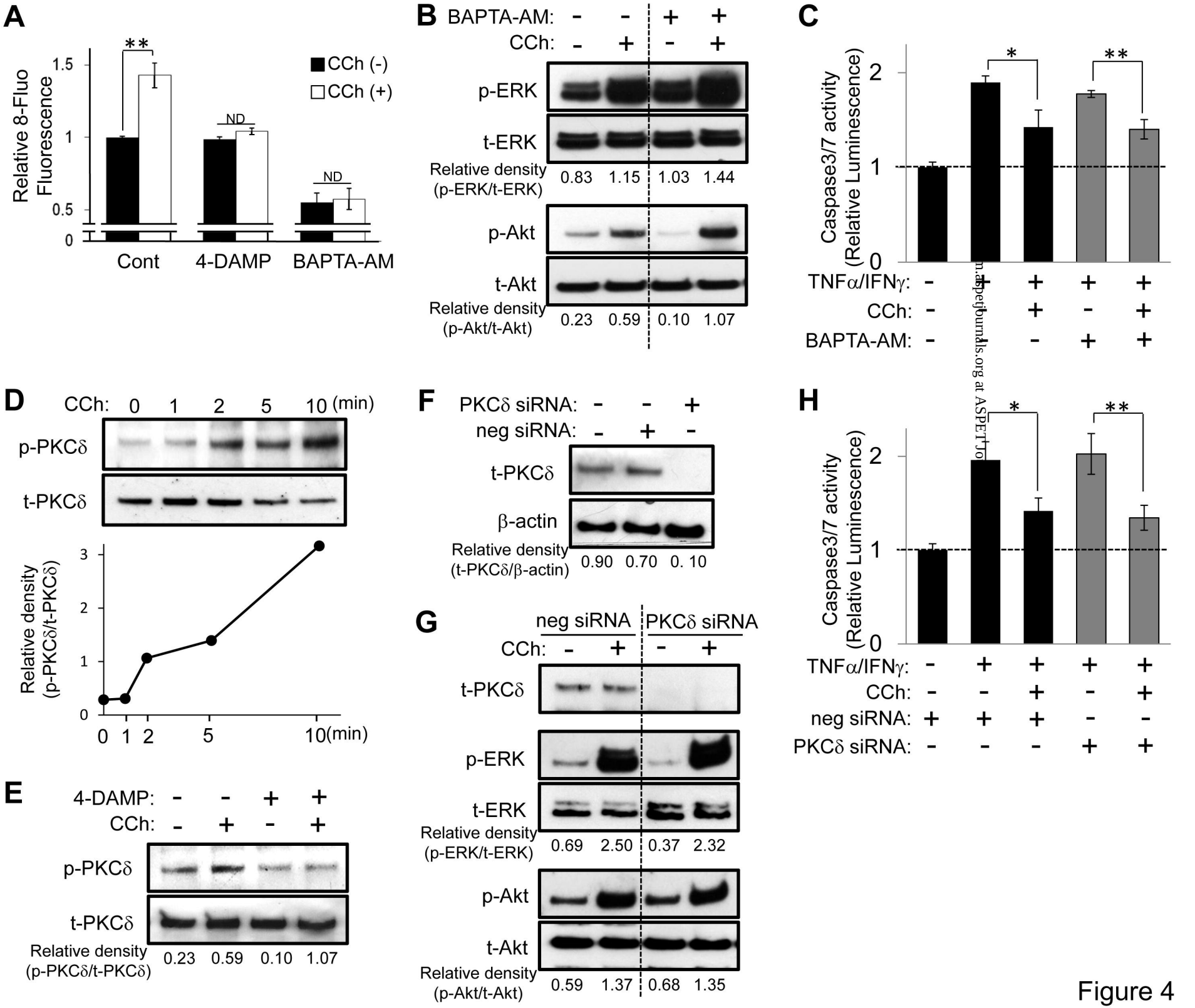


Figure 4

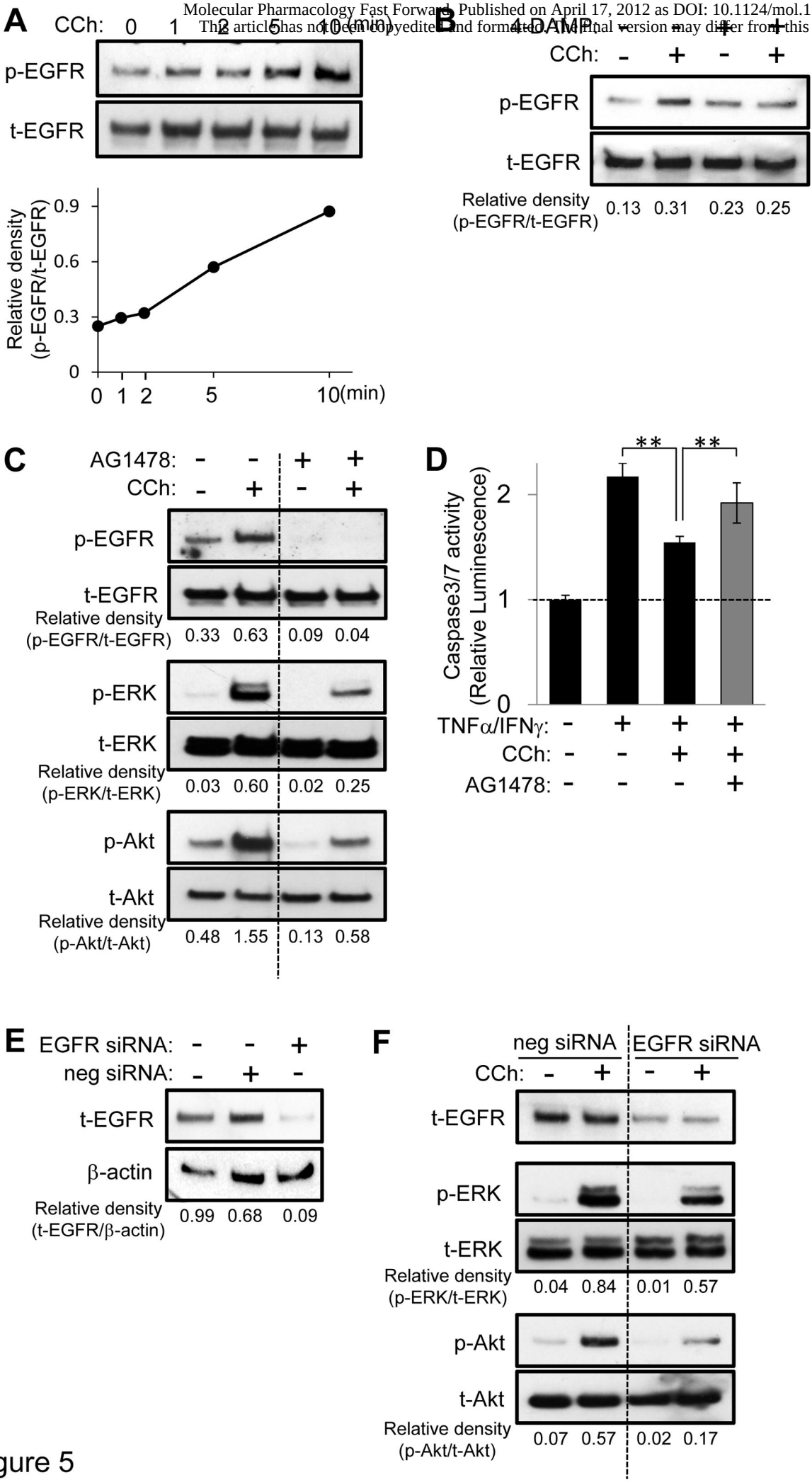


Figure 5

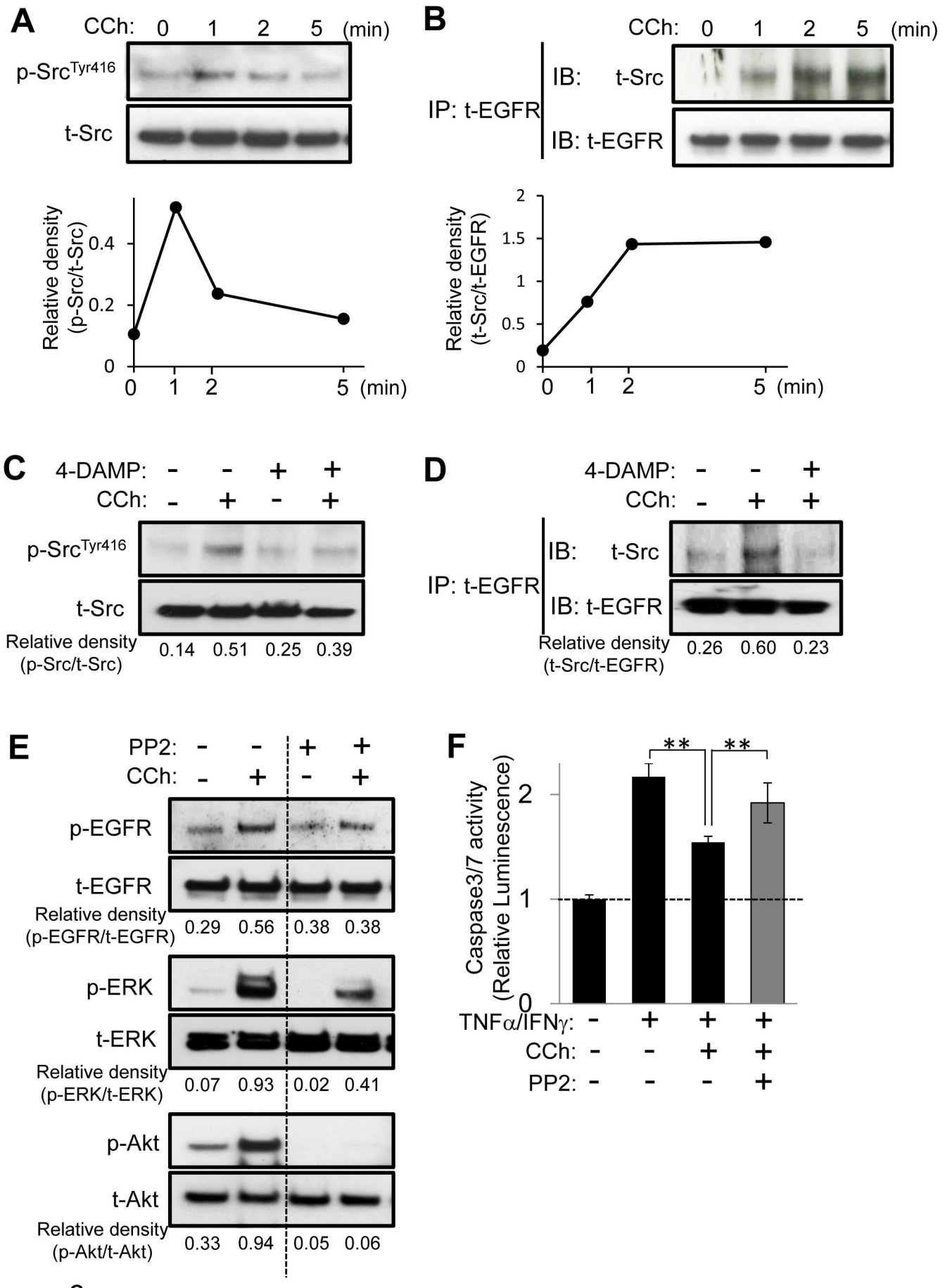

A
CCh: 0 1 2 5 (min)
p-Src^Tyr416
t-Src
Relative density (p-Src/t-Src)
0 0.2 0.4
0 1 2 5 (min)
B
CCh: 0 1 2 5 (min)
IP: t-EGFR
IB: t-Src
IB: t-EGFR
Relative density (t-Src/t-EGFR)
0 0.5 1 1.5 2
0 1 2 5 (min)
C
4-DAMP: - - + +
CCh: - + - +
p-Src^Tyr416
t-Src
Relative density (p-Src/t-Src)
0.14 0.51 0.25 0.39
D
4-DAMP: - - +
CCh: - + +
IP: t-EGFR
IB: t-Src
IB: t-EGFR
Relative density (t-Src/t-EGFR)
0.26 0.60 0.23
E
PP2: - - + +
CCh: - + - +
p-EGFR
t-EGFR
Relative density (p-EGFR/t-EGFR)
0.29 0.56 0.38 0.38
p-ERK
t-ERK
Relative density (p-ERK/t-ERK)
0.07 0.93 0.02 0.41
p-Akt
t-Akt
Relative density (p-Akt/t-Akt)
0.33 0.94 0.05 0.06
F
Caspase3/7 activity (Relative Luminescence)
0 1 2
** **
TNFα/IFNγ: - + + +
CCh: - - + +
PP2: - - - +

Figure 6

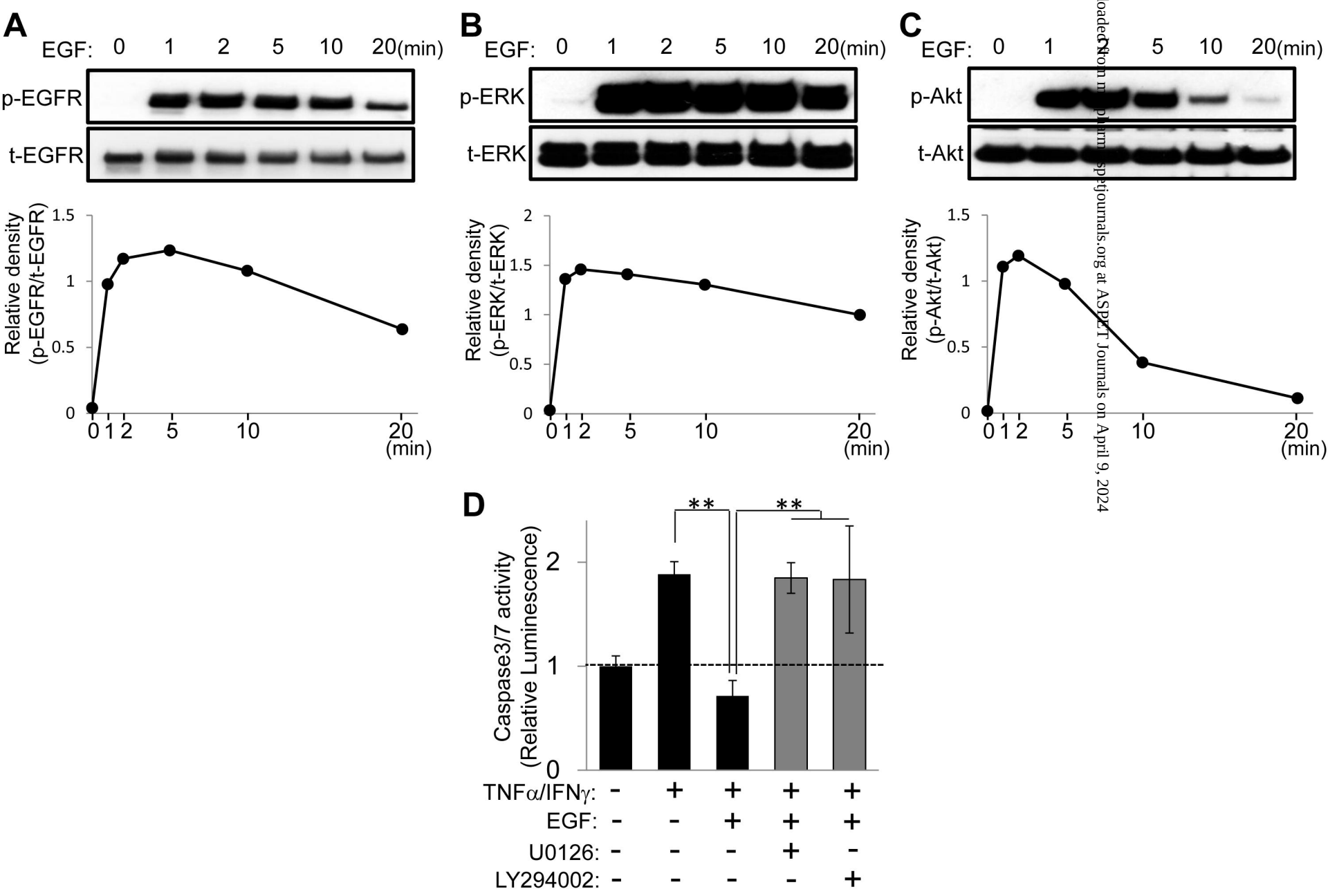


Figure 7

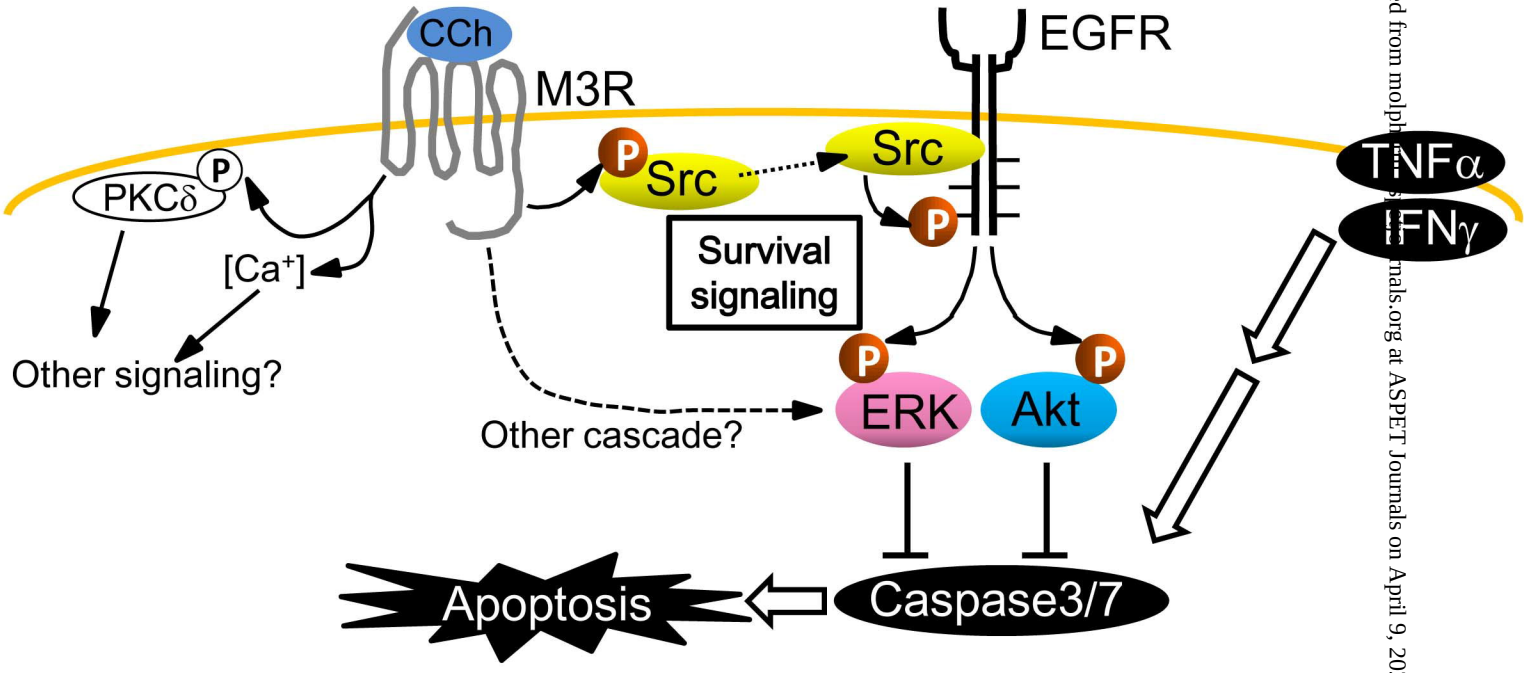
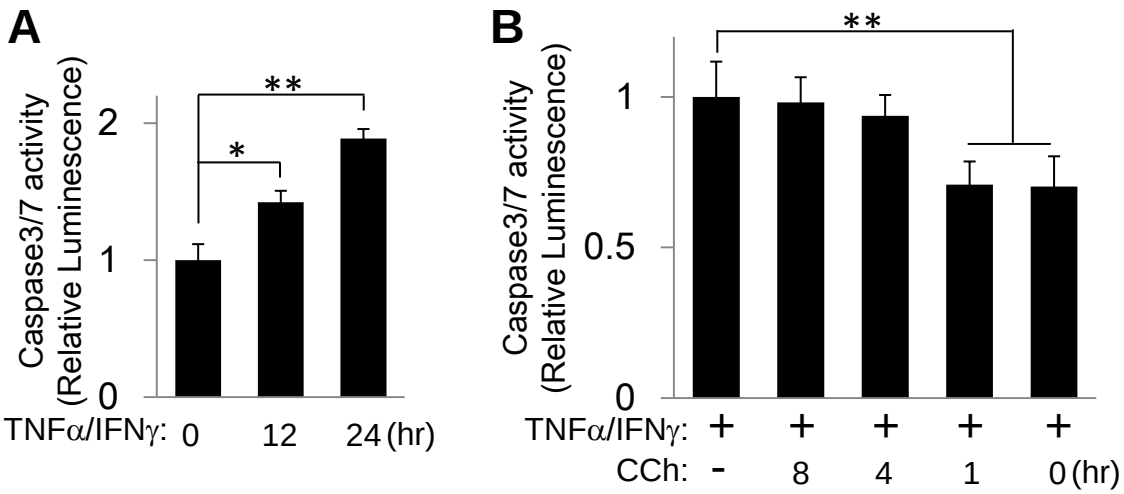


Figure 8

Muscarinic type 3 receptor induces cytoprotective signaling in salivary gland cells via EGFR transactivation

Mikihito Kajiya, Isao Ichimonji, Christine Min, Tongbo Zhu, Jin Jun-O, Qing Yu, Soulafa Almazrooa, Seunghee Cha, and Toshihisa Kawai

Molecular Pharmacology



Supplemental Fig. 1. Relation between proinflammatory cyokint and CCh on caspase3/7 activity.

(A) HSG cells were exposed to TNFα (50 ng/ml)/IFNγ (10 ng/ml) for 0, 12, or 24 hr. (B) HSG cells were pretreated with or without CCh (100 μM) for the indicated time. Then the cells were stimulated with TNFα (50 ng/ml)/IFNγ (10 ng/ml) for 24 hr. Caspase3/7 activity was indicated by luminescence activity. Values represent means ± S.D. of three cultures. * $p < 0.05$, ** $p < 0.01$: Values differ significantly (*t*-test).

Supplemental Figure1

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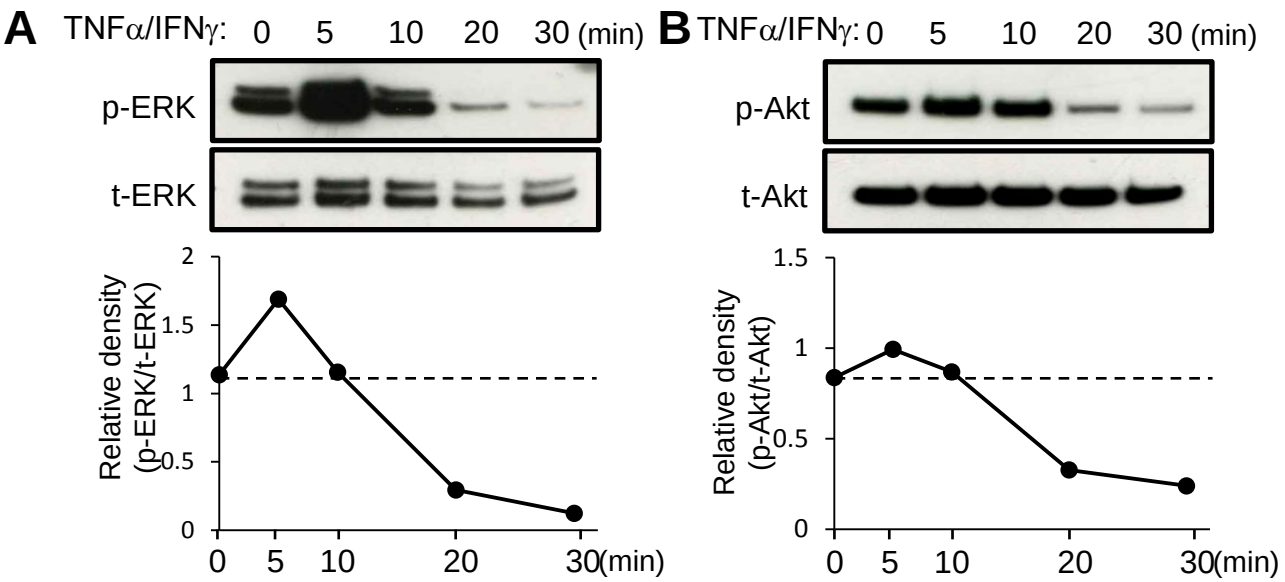

Supplemental Fig. 2. The effect of TNFα/IFNg treatment on ERK and Akt phosphorylation level in HSG cells.

(A and B) HSG cells were exposed to TNFα (50 ng/ml)/IFNγ (10 ng/ml) for the indicated times. The phosphorylated or total ERK (A) and Akt (B) levels were analyzed by immunoblotting. Quantification of the band density was performed by densitometric scanning of each band using NIH image software.

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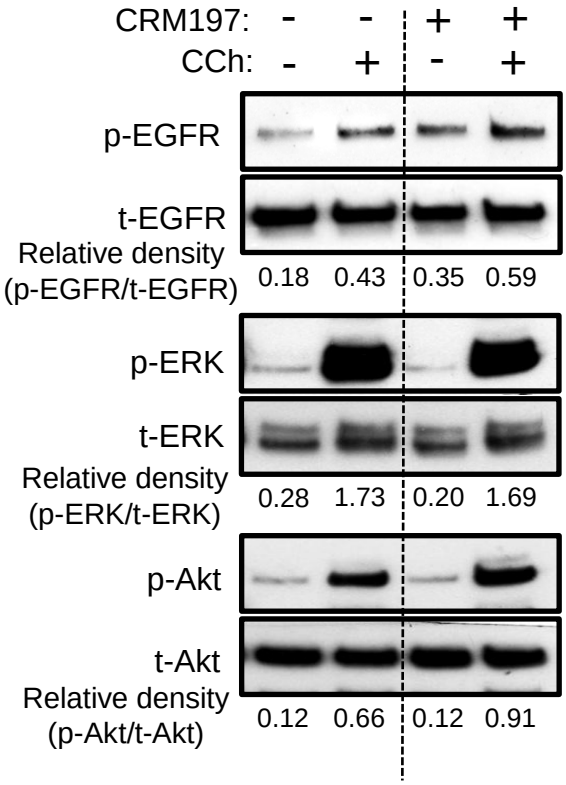

Supplemental Fig. 3. Heparin binding (HB)-EGF inhibitor, CRM197 does not affect the CCh-induced cell survival signaling in HSG cells.

HSG cells were pretreated with or without 1 μg/ml of CRM197 for 30 min and then exposed to CCh (100 μM) for 5 min. The phosphorylated or total EGFR, ERK, and Akt levels were analyzed by immunoblotting.

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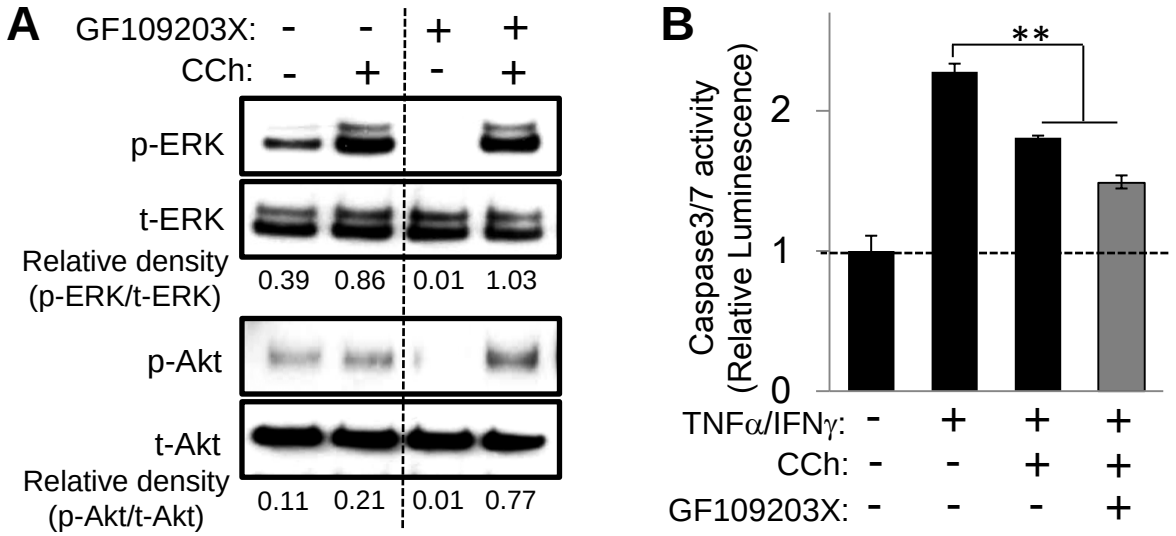

Supplemental Fig. 4. PKC inhibitor, GF109203X does not affect the CCH-induced cell survival signaling in HSG cells.

(A) HSG cells were pretreated with or without GF109203X (1 μM) for 30 min and then exposed to CCh (100 μM) for 5 min. The phosphorylated or total ERK and Akt levels were analyzed by immunoblotting. Quantification of the band density was performed by densitometric scanning of each band using NIH image software. (B) HSG cells were pretreated with or without GF109203X (1 μM) for 30 min. Then the cells were treated with or without 100 μM of CCh in the presence or absence of combined TNFα (50 ng/ml)/IFNγ (10 ng/ml) and incubated for 24 hr. Caspase3/7 activity was indicated by luminescence activity as described in the Materials and Methods section. Values represent means ± S.D. of three cultures. ** $p < 0.01$: Values differ significantly (*t*-test).