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# Involvement of membrane-type bile acid receptor M-BAR/TGR5 in bile acid-induced activation of epidermal growth factor receptor and mitogen-activated protein kinases in gastric carcinoma cells ☆

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**Abstract**

Bile acids, which have been implicated in gastrointestinal-tract cell carcinogenesis, share properties with tumor promoters in that both affect signal transduction pathways responsible for cell proliferation and apoptosis. In the present study, we demonstrate that EGFR–ERK1/2 is activated following treatment of AGS human gastric carcinoma cells with bile acids. EGFR phosphoactivation is ligand-dependent, since treatment of cells with HB–EGF antisera or CM197 (a selective inhibitor of HB–EGF) markedly inhibits deoxycholate (DC)-promoted activation. Membrane-type bile acid receptor (M-BAR)/TGR5 is a recently identified G-protein-coupled receptor (GPCR). In AGS cells, siRNAs that target M-BAR suppress DC-induced phosphorylation of EGFR. Furthermore, introduction of siRNAs targeting ADAM17 transcripts resulted in suppression of DC-induced activation of EGFR and ERK1/2. These results suggest that in AGS cells, DC transactivates EGFR through M-BAR- and ADAM/HB–EGF-dependent mechanisms.


*Keywords:* Bile acids; EGF receptor; Transactivation; M-BAR

Epidemiologic and experimental observations in model systems have implicated bile acids in gastrointestinal-tract cell carcinogenesis [1]. Duodenogastric reflux is associated with gastric and esophageal carcinogenesis, in that increased reflux after distal gastrectomy increases the risk of gastric cancer [2]. In animal models, bile reflux induces gastric and esophageal adenocarcinoma [3]. Several reports suggest that the cellular effects of bile acids involve activation of mitogen-activated protein kinase (MAPK) and epidermal growth factor (EGFR) receptors [4–6]. Although the mechanism of bile acid-induced activation of EGFR in gastrointestinal cells is currently not well understood, it is likely to stimulate cell proliferation, inhibit apoptosis and elevate levels of cyclooxygenase-2 (COX-2) expression [6–9], as occurs in other human carcinomas.

Accumulating evidence implicates various extracellular stimuli unrelated to EGFR ligands, such as agonists of G-protein-coupled (GPCR) and cytokine receptors [10,11], in the transactivation of EGFR. The EGF ligand family

*Abbreviations:* GPCR, G-protein-coupled receptor; M-BAR, membrane-type bile acid receptor; DC, deoxycholate; ERK, extracellular signal regulated kinase; EGF, epidermal growth factor; HB–EGF, heparin-binding EGF-like growth factor, ADAM, a disintegrin and metalloproteinase.

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consists of EGF, heparin-binding EGF-like growth factor (HB–EGF), transforming growth factor (TGF)-α, epiregulin, amphiregulin, neuregulins and betacellulin [12]. EGFR transactivation requires activation of disintegrin and the metalloproteinase ADAM, which induce the shedding of EGF-ligand ectodomains during their transition to soluble bioactive factors [13]. Many GPCR agonists appear to mediate EGFR transactivation by activating metalloprotease-dependent HB–EGF shedding [14,15]. A recent search for the ligand to an orphan GPCR resulted in the identification of a novel GPCR known as membrane-type bile acid receptor (M-BAR), or TGR5/BG37 [16,17]. Although the physiological function of M-BAR is not yet well understood, M-BAR has been shown to transduce bile-acid signals independent of a nuclear receptor-mediated response. Expression of M-BAR has been detected in most human tissues, including stomach and colon.

In the present study, we examined how bile acids activate EGFR–ERK1/2 signaling in the human gastric carcinoma cell line AGS. We demonstrate that transactivation of EGFR by M-BAR occurs via an HB–EGF-dependent mechanism with specific involvement of ADAM metalloproteinase. These results suggest that regulation of M-BAR inhibition could lead to new strategies for prevention of upper-gastrointestinal carcinogenesis.

## Experimental procedures

*Materials.* Antibodies against EGFR, phospho-specific antibodies against $Tyr^{1068}$-phosphorylated EGFR, and phospho- and non-specific antibodies to ERK1/2 were purchased from Santa Cruz Biotechnology (Santa Cruz). EGFR-neutralizing antibody (Clone 225) was purchased from Lab Vision Corporation. Amphiregulin- and HB–EGF-neutralizing antibodies were purchased from R&D. TGFα-neutralizing antibody was purchased from BioVision. Antibodies against ADAM10 and ADAM17 were purchased from AnaSpec Inc. Antibody against ADAM12 was from Abcam. PD168393, CRM197 and 2R-[(4-biphenylsylfonyl) amino]-*N*-hydroxy-3-phenylpropinamide (BiPS) were purchased from Calbiochem.

*Cell culture.* AGS, a well-differentiated human gastric adenocarcinoma cell line, was provided by the ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 20 mM Hepes, 10 mM $NaHCO_3$ and antibiotics. All the cultures were incubated at 37 °C under 95% air and 5% $CO_2$.

*Immunoblot analysis.* Cell lysates were prepared as described previously [6]. For the analysis of EGFR, cells were washed once with PBS and then lysed for 10 min on ice in buffer containing 50 mM Hepes, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, 10 mM sodium pyrophosphate, 2 mM sodium orthovandate, 10 mM sodium fluoride, 1 mM PMSF and 10 μM/mL aprotinin, and precleared by centrifugation at 12,500*g* for 10 min at 4 °C. Lysates were sonicated for 20 s on ice and centrifuged at 10,000*g* for 10 min. Proteins (25 μg/lane) were resolved by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and then transferred to nitrocellulose membranes. Membranes were exposed to primary antibodies overnight at 4 °C. Immunoreactive proteins were visualized using the enhanced chemiluminescence (ECL) detection system (Amersham Pharmacia Biotech).

*RT-PCR for HB–EGF, TGFα, amphiregulin, and M-BAR.* Total RNA was extracted from the cells using TRIzol reagent (Invitrogen). First-strand cDNA prepared from total RNA using Moloney Murine Leukemia Virus RT and oligo(dT) random primers, was used as template for amplification of gene-specific double-stranded cDNAs by *Taq* polymerase (Invitrogen) in a reaction mixture containing 10 pmol of each primer set: HB–EGF, 5′-GGGCATGACTAATTCCCACTGA-3′and 5′-GCCCAATCCTAGACGGCAAC-3′; TGFα, 5′-AGATAGACAGCAGCCAACCCTGA-3′, 5′-CTAGGGCCATTCTGCCCATC-3′, amphiregulin, 5′-GTGGTGCTGTCGCTCTTGATACTC-3′, 5′-TCAAATCCATCAGCACTGTGGTC-3′; M-BAR, 5′-GGAGCGGGCAGTGTGCCGCGATGAG-3′, 5′-TTAGTTCAAGTCCAGGTCGACACTGCTTT-3′. PCR products resolved by electrophoresis in 2% agarose gels were detected by ethidium bromide staining.

*Preparation and transfection of short interfering RNA oligonucleotides.* The sequences of complementary pairs of sense/antisense 25-base ribonucleotides corresponding to specific genes are as follows: 5′-ACC UGU ACC U CG AAG UCUAUG GGCU-3′ and 5′-AGC CCA UAG ACU UCG AGG UAC AGGU-3′ for M-BAR; 5′-CAU CUG GGG ACA AAC UUA ACA ACAA-3′ and 5′-UUG UUG UUA AGU UUG UCC CCA GAUG-3′ for ADAM10; 5′-GUU UCA GAG GCA AGG AAA AGA UCUG-3 and 5′-CAG AUC UUU UCC UUG CCU CUG AAAC-3′ for ADAM12; 5′-GGU GGA UGG UAA AAA CGA AAG CGAG-3′ and antisense 5′-CUC GCU UUC GUU UUU ACC AUC CACC-3′ for ADAM17. The oligonucleotides were synthesized by iGENE. AGS cells were transfected with siRNAs using Transfectamine Reagent (Ambion) according to the manufacturer's instruction. All functional assays were performed two days after transfection.

*Apoptosis assay.* Apoptosis was assessed using the Cell-Death Detection ELISA kit (Roche Molecular Biochemicals), which measures the level of soluble histone–DNA complexes that accumulate during DNA fragmentation. Cells seeded on 24-well plates at a density of $2 \times 10^4$ cell/well were starved for serum and then treated with specific test reagents for 3 h. ELISAs were performed according to the manufacturer's instructions.

*Statistical Analyses.* Results were expressed as means ± SD, and statistical significance was determined using Student's *t*-test for paired data. Differences of $P < 0.05$ were considered significant.

## Results

### *DC-induced ERK1/2 activation in AGS cells*

DC induces a rapid activation of ERK1/2 in many different cell systems including liver [4], biliary [5], and gastric cells [6]. Consistent with previous findings, treatment of AGS cells with DC-induced phosphorylation of ERK-1/2 MAPK within 5 min that was detectable for at least 2 h (Fig. 1a).

### *DC activation of EGFR in AGS cells is ligand-dependent*

Previous studies have implicated EGFR activation in bile acid-mediated activation of ERK1/2 in hepatocytes and cholangiocytes [4,5]. We tested whether EGFR was also involved in the DC-induced activation of ERK1/2 in AGS cells. As shown in Fig. 1a, AGS cells treated with DC show a specific increase in the relative amount of tyrosine-phosphorylated EGF receptor within 5 min and for at least 2 h.

When AGS cells were treated with PD168393 (a specific inhibitor of EGFR tyrosine kinase) to test whether the phospho-activation of EGFR was causally linked to ERK1/2 activation, we observed potent inhibition of DC-mediated activation of ERK1/2 (Fig. 1b). Pretreatment of AGS cells with anti-EGFR-blocking antibody inhibited the DC-induced phospho-activation of EGFR as well as ERK1/2 (Fig. 2a), suggesting that DC activation of the EGFR–ERK1/2 pathway is ligand-dependent.

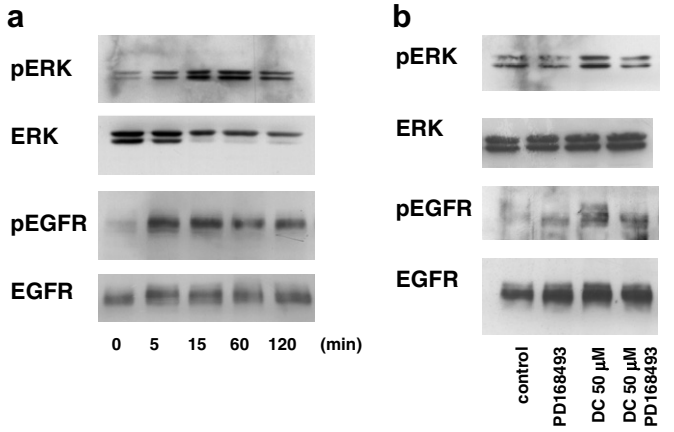


Fig. 1. (a) Effects of DC on ERK1/2 and EGFR phosphorylation in AGS cells. Cells treated with 50 μmol/L of DC were harvested at the indicated time. Cell lysates were immunoblotted with antibodies against phosphorylated ERK1/2 (p-ERK1/2), total ERK1/2, antibodies against $Tyr^{1068}$-phosphorylated EGFR (p-EGFR) and total EGFR as indicated. Results are representative of three independent experiments with similar results. (b) Effects of PD168393 on DC-induced ERK1/2 and EGFR phosphorylation in AGS cells. Lysates of AGS cells treated with 50 μmol/L DC for 5 min after preincubation with or without 10 μmol/L of PD168393 for 1 h were analyzed for their reactivity with antibodies to p-ERK1/2, total ERK1/2, p-EGFR and total EGFR in immunoblots. Results are representative of three independent experiments with similar results.

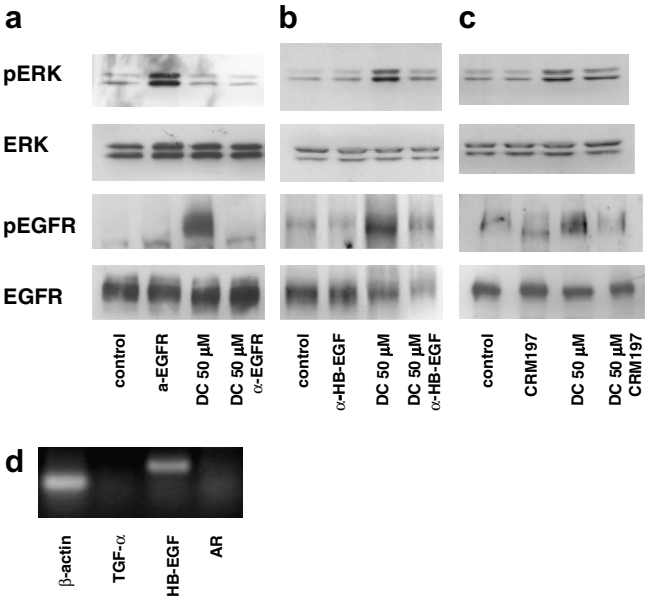


Fig. 2. (a) Effects of anti-EGFR antibody on DC-induced ERK1/2 and EGFR phosphorylation in AGS cells. Cells were treated with 50 μmol/L DC for 5 min after preincubation with or without 1 μg/mL of anti-EGFR antibody (Clone 225) for 1 h. Immunoblot analyses for p-ERK1/2, total ERK1/2, p-EGFR, and total EGFR were performed. Results are representative of three independent experiments with similar results. (b) Cells were treated with 50 μmol/L DC for 5 min after preincubation with or without 1 μg/mL of anti-HB–EGF antibody for 1 h. Immunoblot analysis for p-ERK1/2, total ERK1/2, p-EGFR, and total EGFR were performed to assess the involvement of HB–EGF on DC-induced ERK1/2 and EGFR phosphorylation. (c) Cells were treated with 50 μmol/L DC for 5 min after preincubation with or without a selective inhibitor of HB–EGF, CRM197 (10 μg/mL) for 1 h. Immunoblot analysis for p-ERK1/2, total ERK1/2, p-EGFR, and total EGFR were performed. Results are representative of three independent experiments with similar results. (d) Total cellular RNA was isolated from AGS cells, and RT-PCR was used to detect mRNAs encoding EGFR ligands including transforming growth factor-α (TGFα) and amphiregulin (AR).

### *HB–EGF is involved in DC-induced EGFR and ERK1/2 activation*

To identify the ligand(s) responsible for DC-induced EGFR phosphorylation and ERK1/2 activation, AGS cells were pretreated with neutralizing antibodies against HB–EGF, AR, or TGFα before incubation with DC. Among the antisera tested, anti-HB–EGF antiserum blocked DC-induced EGFR phosphorylation and ERK1/2 activation potently (Fig. 2b), whereas neutralizing antibodies against AR or TGFα had little effect on DC-induced EGFR transactivation (data not shown).

The effect of the diphtheria toxin analogue CM197 (a specific inhibitor of primate HB–EGF [14]) was used to assess the involvement of HB–EGF on the activation of EGFR and ERK1/2 (Fig. 2c). Pretreatment of AGS cells with CM197 markedly inhibited DC-mediated activation of EGFR and ERK1/2. We performed RT-PCR analysis to examine expression of HB–EGF in AGS cells and found that transcripts of HB–EGF were readily amplified (Fig. 2d). These observations suggested that DC-mediated EGFR activation is ligand-dependent, and that HB–EGF can function as a ligand for bile acid-induced EGFR phosphorylation in AGS cells.

### *Involvement of GPCR on DC-induced EGFR tyrosine phosphorylation and ERK1/2 activation*

We examined whether GPCR was involved in DC-induced EGFR transactivation in AGS cells. It has been reported that bile acids such as lithocholyltaurine and lithocholylglycine, which have similar molecular structure to acetylcholine, interact with muscarinic receptors [18]. Pretreatment of AGS cells with the muscarinic receptor antagonist atropine did not alter DC-mediated activation of EGFR or ERK1/2 significantly (Fig. 3a). Recently, a novel GPCR responsive to bile acids and termed M-BAR was identified during a search for an orphan GPCR ligand. M-BAR interacts with a broad range of bile acids, including DC, when expressed in transfected cells [16,17]. M-BAR's involvement in DC-induced EGFR activation was tested by transfection of M-BAR-specific siRNAs into AGS cells. Significant and specific knockdown of endogenous M-BAR mRNA levels was observed (Fig. 3b), and the cells treated with M-BAR siRNAs also showed potent suppression of DC-induced EGFR tyrosine phosphorylation and ERK1/2 activation (Fig. 3c). These results suggest that DC-induced activation of EGFR in AGS cells involves the activity of M-BAR/TGR5.

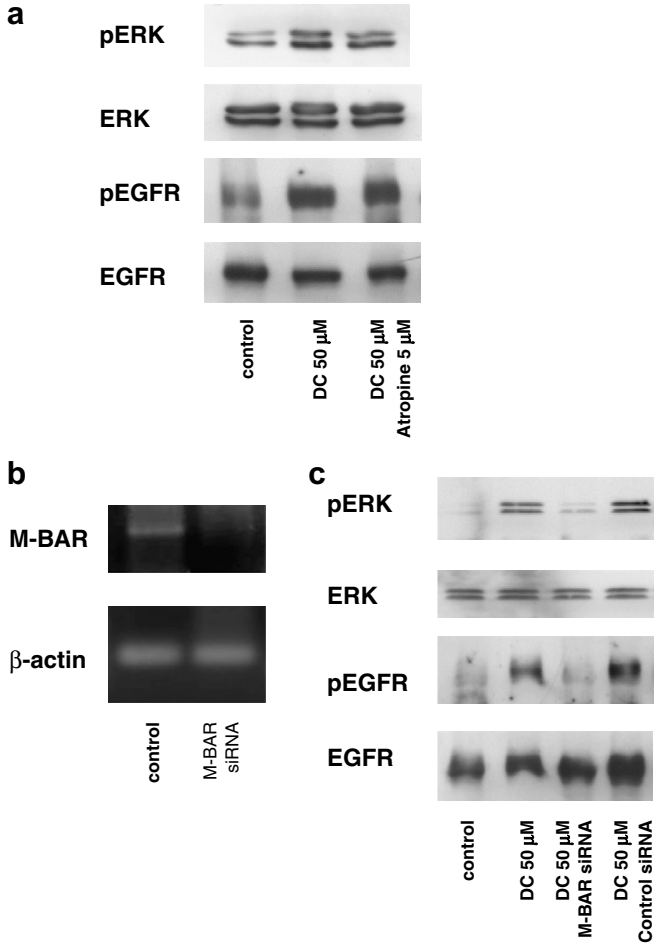


Fig. 3. (a) Effects of the muscarinic antagonist atropine on DC-induced ERK1/2 and EGFR phosphorylation in AGS cells. Cells were treated with 50 μmol/L DC for 5 min after preincubation with or without 5 μmol/L of atropine for 30 min. Immunoblots of AGS cell extracts were performed to analyze the expression of p-ERK1/2, total ERK1/2, p-EGFR, and total EGFR. Results are representative of three independent experiments with similar results. (b) Effects of knockdown of M-BAR mRNA by the specific siRNA. Cells were treated with siRNA that targets M-BAR, and its effect on M-BAR mRNA was analyzed using RT-PCR. (c) Effects of M-BAR knockdown on DC-induced ERK1/2 and EGFR phosphorylation. Cells were treated with 50 μmol/L DC for 5 min after exposure to siRNA targeting M-BAR. AGS cells lysates were analyzed in Western immunoblots with antibodies specific to p-ERK1/2, total ERK1/2, p-EGFR, and total EGFR. Results are representative of three independent experiments with similar results.

### *Effects of ADAM siRNA on DC-induced EGFR tyrosine phosphorylation and ERK1/2 activation*

Many GPCR agonists have been reported to mediate EGFR transactivation via metalloprotease-dependent HB–EGF shedding. To test the involvement of metalloproteases on DC-induced EGFR activation, AGS cells were treated with the metalloprotease inhibitor, BiPS, which is known to inhibit EGFR transactivation by angiotensin II [14,19]. Pretreatment of AGS cells with BiPS inhibited DC-induced EGFR activation potently (Fig. 4a). Since several ADAM-family metalloproteases, including ADAM10, 12, and 17, have been implicated in HB–EGF shedding [14,20,21]. We used siRNAs to knockdown specific endogenous ADAM proteins and then tested for the effect of these knockdowns on the DC-dependent activation of EGFR and ERK1/2. Although significant and specific knockdown of endogenous protein levels was confirmed in cells transfected with ADAM 10, ADAM 12 or ADAM 17 siRNAs (Fig. 4b), only ADAM17-specific siRNAs suppressed DC-induced EGFR and ERK1/2 activation (Fig. 4c). Transfection of ADAM 10, 12, or 17-specific siRNAs had no measurable effect on the basal phosphorylation state of ERK1/2 or EGFR (data not shown).

### *Inhibition of EGFR transactivation potentiates DC-induced apoptosis*

Using AGS cells, we investigated the role of EGFR transactivation in DC-induced cell death. As shown in Fig. 4d, DC-induced AGS cell apoptosis (measured by ELISA) was not altered in the presence of EGF; however, inhibition of EGFR tyrosine kinase with PD168393 potentiated DC-induced apoptosis significantly. Furthermore, both AGS cells treated with the ADAMs metalloprotease inhibitor BiPS or transfected with M-BAR specific siRNA showed potentiation of DC-induced apoptosis. Addition of EGF to cultured cells attenuated the effect of BiPS or M-BAR-specific siRNA on DC-induced apoptosis. These results indicate that EGFR transactivation can partially protect AGS cells from DC-induced apoptosis.

## Discussion

We report that EGFR–ERK1/2 pathways are activated in AGS cells treated with DC. Our results suggest that the novel GPC receptor M-BAR functions in the transactivation event, and that transactivation of EGFR, specifically, occurs via an ADAM/HB–EGF dependent pathway. Our results provide new insights that may contribute to the development of therapeutic tools to treat or prevent gastrointestinal neoplasm.

Data from many reports make it clear that the EGF receptor functions within signaling networks that are activated by stimuli, including GPCR agonists, cytokines, and chemical and environmental stressors [10,11,14,22–24], that do not act directly on the EGFR. Although it was originally reported that bile acid-induced EGFR activation in hepatocytes is ligand independent [4], Werneburg et al. reported that a TGFα dependent mechanism for DC-induced activation of EGFR existed in human cholangiocyte cell lines [5]. The data in this study indicate that DC-induced activation of EGFR occurs via an HB–EGF-dependent mechanism in AGS cells. The differential reactivity of antibodies used in these studies may account for at least some of the differences in these findings.

Nuclear receptors including the farnesoid X receptor (FXR) [25] have been identified as bile acid receptors that regulate the transcription of genes encoding pivotal bile

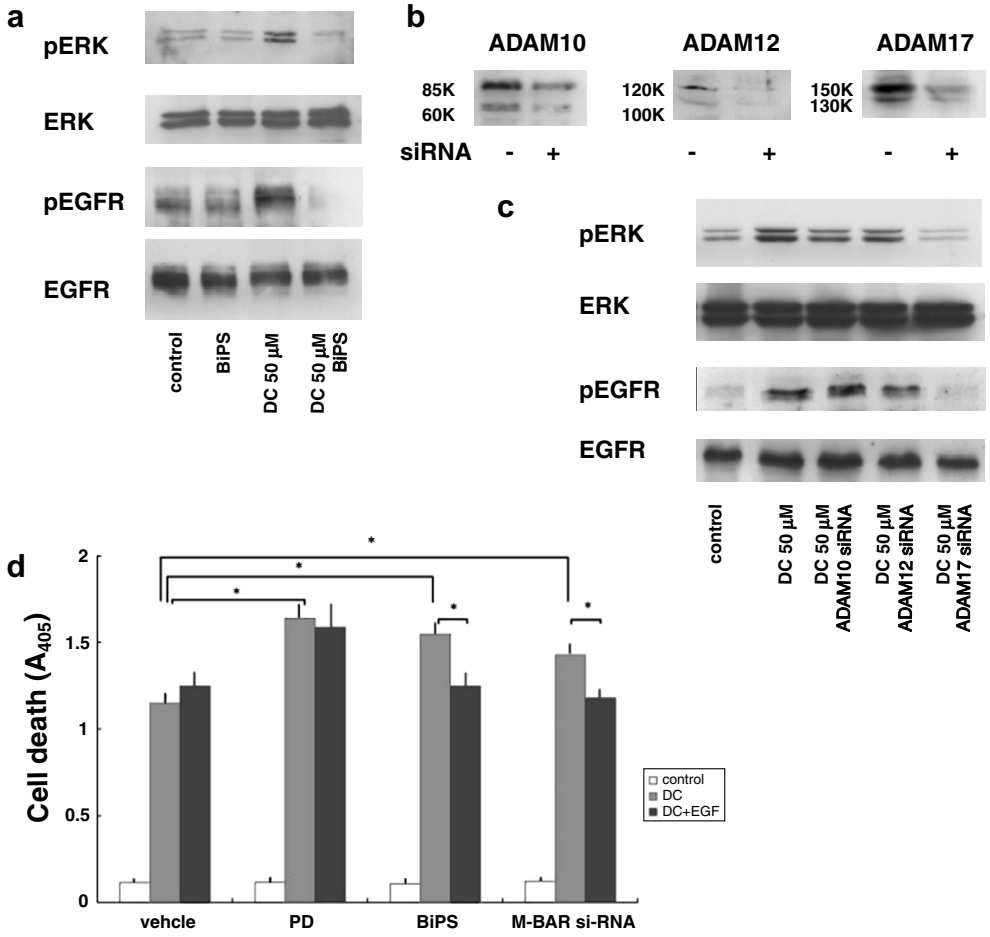


Fig. 4. (a) Effect of metalloprotease inhibitor on the DC-induced phosphorylation of EGFR and ERK1/2 in AGS cells. Cells were treated with 50 μmol/L of DC for 5 min after preincubation with or without 10 μmol/L of BiPS for 1 h. AGS cell extracts were analyzed by Western immunoblots with antibodies specific to p-ERK1/2, total ERK1/2, p-EGFR, and total EGFR. Results are representative of three independent experiments with similar results. (b) Knockdown of endogenous ADAM protein by siRNAs was assessed by immunoblotting with antibodies against respective ADAM proteins. (c) Effects of siRNA knockdown of ADAMs on DC-induced EGFR and ERK1/2 phosphorylation in AGS cells. Cells were treated with siRNAs targeting ADAM10, 12, or 17. AGS cell lysates were analyzed by immunoblotting for the presence of p-ERK1/2, total ERK1/2, p-EGFR, and total EGFR. Results are representative of three independent experiments with similar results. (d) Effect of EGFR activation on DC-induced AGS cell apoptosis. Cells were treated with 50 μmol/L DC for 3 h after preincubation with or without PD168393 (10 μmol/L), BiPS (10 μmol/L), EGF (5 nM/L) for 1 h, or after pretreatment with control or M-BAR-specific siRNAs. DC-induced apoptosis of AGS cells was assessed using the Cell-Death Detection ELISA. $^{*}P < 0.05$ versus DC + vehcle or DC + control si-RNA. Results are representative of three independent experiments performed in duplicate with similar results.

acid biosynthetic enzymes and transport proteins. The rapid activation of EGFR or ERK1/2 following bile acid treatment makes it unlikely that activation occurs in response to altered transcription of nuclear bile-acid receptors. Alternatively, agonists of GPCRs, cytokines and stressors have been shown to induce HB–EGF ectodomain shedding via activation of ADAM [13–15,21,26]. Taurine- and glycine-conjugated bile acids, which have similar molecular to acetylcholine, have been reported to activate the M3 muscarinic receptor and to transactivate EGFR [18,27]. M-BAR/TGR5/BG37 is a recently identified GPCR that is responsive to bile acids [15,16]. In CHO cells expressing M-BAR, treatment with bile acids induced intracellular cAMP production and activation of ERK1/2. M-BAR is also involved in bile acid-induced GLP secretion in intestinal neuroendocrine cells [28] and in bile acid-induced thyroid hormone activation [29]. Here, we demonstrate that M-BAR is involved in DC-induced EGFR activation in AGS cells.

Bile-acid mediated activation of EGFR stimulates COX-2 expression gastrointestinal cells [5–7]. COX-2, which catalyzes the synthesis of prostaglandins (PG) from arachidonic acid, is known to be overexpressed in many cancers [8,9] and may contribute to epithelial cell carcinogenesis by inhibiting apoptosis, promoting growth, and supporting angiogenesis. Bile acids induce COX-2 expression in AGS cells and human cholangiocytes via the EGFR–ERK1/2 pathway [4,5]. The present study indicates that EGFR transactivation partially protects AGS cells from DC-induced apoptosis. Taken together with our observations in gastric AGS cells, these findings lead us to hypothesize that M-BAR participates in the genesis and apoptosis of gastrointestinal neoplasm, and thus may serve as a target for cancer prevention.

In summary, these experiments demonstrate that M-BAR is involved in bile acid-induced EGFR transactivation in gastric AGS cells, and that DC-induced transactivation of the EGFR is dependent on ADAM/HB–EGF-mediated signaling. These data support further investigations targeting the therapeutic potential of M-BAR in the treatment and prevention of GI carcinogenesis.

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