


RESEARCH ARTICLE

Overexpression of sigma-1 receptor inhibits ADAM10 and ADAM17 mediated shedding *in vitro*

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ABSTRACT

The sigma-1 receptor is a molecular chaperone protein highly enriched in the brain. Recent studies linked it to many diseases, such as drug addiction, Alzheimer's disease, stroke, depression, and even cancer. Sigma-1 receptor is enriched in lipid rafts, which are membrane microdomains essential in signaling processes. One of those signaling processes is ADAM17- and ADAM10-dependent ectodomain shedding. By using an alkaline phosphatase tagged substrate reporter system, we have shown that ADAM10-dependent BTC shedding was very sensitive to both membrane lipid component change and sigma-1 receptor agonist DHEAS treatment while ADAM17-dependent HB-EGF shedding was not; and overexpression of sigma-1 receptor diminished ADAM17- and ADAM10-dependent shedding. Our results indicate that sigma-1 receptor plays an important role in modifying the function of transmembrane proteases.

KEYWORDS sigma-1 receptor, ADAM17, ADAM10, shedding, lipid raft

INTRODUCTION

The sigma-1 receptor is a molecular chaperone protein with two transmembrane domains and it is highly enriched in the brain (Hayashi and Su, 2007, 2010). It was first identified by Su in 1982 as a subtype of opioid receptors that bind to SKF10047 (N-allylnormetazocine) (Su, 1982) and cloned in 1996 (Hanner et al., 1996; Seth et al., 1998; Mei and Pasternak, 2001). However, it is now clear that sigma-1 receptor is a unique protein that is different from opioid receptors and it can bind to steroids such as cholesterol,

progesterone, and DHEAS (dehydroepiandrosterone) (Maurice and Su, 2009; Hayashi and Su, 2010). Besides those neurosteroids, sigma-1 receptor also interacts with drugs such as cocaine and methamphetamine (Sharkey et al., 1988; Itzhak, 1993). Such profits have linked sigma-1 receptor to many diseases, such as drug addiction, Alzheimer's disease, stroke, and depression (Maurice and Su, 2009). Sigma-1 receptor is also found to be highly expressed in many cancer cells (Vilner et al., 1995) and involved in regulating cancer cell proliferation by modifying lipid rafts (Palmer et al., 2007)

Lipid rafts are membrane microdomains that are essential in signaling processes (Palmer et al., 2007; Staubach and Hanisch, 2011). Sigma-1 receptor is enriched with lipid raft markers caveolin-2 (Hayashi and Su, 2003, 2007; Hayashi and Fujimoto, 2010) and they can modify lipid fluidity by remodeling lipid rafts (Hayashi and Su, 2005, 2010; Palmer et al., 2007). Overexpressing sigma-1 receptor in PC12 cells caused the reconstitution of lipid rafts by changing the relative distribution of cholesterol between the raft and nonraft fractions without changing the total concentration of cholesterol (Takebayashi et al., 2004). Later, Palmer et al. showed that cholesterol binds to cholesterol recognition domain in the C-terminus of sigma-1 receptors, which is also part of the sigma-1 receptor drug binding domain. In addition, both cholesterol depletion with methyl-cyclodextrin and SKF10047 binding can increase the amount of sigma-1 receptor in lipid raft fractions in breast cancer cell lines (Palmer et al., 2007).

ADAM17 (a disintegrin and metalloprotease 17) and ADAM10 (a disintegrin and metalloprotease 10) are important α -secretase responsible for the non-amyloidogenic processing of the Alzheimer's diseases (AD)-associated amyloid precursor protein (APP) (Pruessmeyer and Ludwig, 2009). Those two transmembrane proteases are also involved in the

shedding process of many other substrates, such as ligands of the epidermal growth factor receptor (EGFR), Notch1, L1 cell adhesion molecule (L1CAM), which are involved in pathological processes and human diseases such as cancer and stroke (Pruessmeyer and Ludwig, 2009). It is now known that lipid rafts can affect both ADAM17 and ADAM10 function as cholesterol depletion can trigger both ADAM10-mediated CD44 shedding (Murai et al., 2011) and ADAM17-mediated CD30 shedding (von Tresckow et al., 2004), collagen XVII shedding (Zimina et al., 2005), and TNF shedding (Tellier et al., 2006, 2008). It has been reported that ADAM10 is located in the nonraft region of the membrane of neuroblastoma SH-SY5Y cells when functioning as α -secretase of APP (Harris et al., 2009) and it cannot cleave APP in a cholesterol-rich environment (Kojro et al., 2010). Overexpressing caveolin-1 can markedly inhibit angiotensin II-induced ADAM17-dependent EGFR ligand shedding (Takaguri et al., 2011). Therefore, both metalloproteases' function can be modified by lipid raft.

Given the circumstances that those metalloproteases' function can be affected by lipid raft which can be modified by sigma-1 receptor, it is very likely that sigma-1 receptor may also affect the metalloproteases' function. By using an alkaline phosphatase tagged substrate reporter system, we have shown that overexpression of sigma-1 receptor can diminish ADAM17- and ADAM10-dependent shedding. Interestingly, our system revealed that ADAM10-dependent shedding is more sensitive to lipid modification while ADAM17-dependent shedding is not. Overexpression of sigma-1 receptor can inhibit such sensitivity as neither additional cholesterol nor cholesterol depletion has any further effect on ADAM17- or ADAM10-dependent shedding. Our results indicate an important role of sigma-1 receptor in modifying cell membrane components and functions.

RESULTS

Overexpressing sigma-1 receptor diminished both PMA induced ADAM17-dependent HB-EGF shedding and ionomycin-induced ADAM10-dependent BTC shedding

To test the protease activity of ADAM17, we used heparin-binding EGF-like growth factor (HB-EGF), a known substrate of ADAM17 (Sahin et al., 2004; Pruessmeyer and Ludwig, 2009), in our shedding assay. PMA (phorbol 12-myristate 13-acetate) is a known activator of ADAM17 through protein kinase C (PKC) activation (Zheng et al., 2002, 2004; Sahin et al., 2004). Therefore, we used PMA induced HB-EGF shedding to check whether ADAM17-dependent shedding is affected by sigma-1 receptors or not. COS-7 or 293T cells were co-transfected with pAPtag5-HB-EGF and pcDNA3-flag-sigma1R expressing constructs or with pAPtag5-HB-EGF and control pcDNA3 constructs and subjected to shedding assay as described previously (Sahin et al., 2004). In brief, the cells were washed by PBS and then incubated in 1 mL OptiMem media for 1 h at 37°C. Then the medium was collected and 1 mL fresh OptiMem with 25 ng/mL PMA was added to cells. The medium was also collected after 1 h incubation. Then all collected supernatants were subjected to colorimetric assay. As shown in Fig. 1, 25 ng/mL PMA can induce ADAM17-dependent HB-EGF shedding as previously reported (Sahin et al., 2004) and this induced ADAM17-dependent HB-EGF shedding was largely reduced by overexpressing sigma-1 receptors in both COS-7 cells (Fig. 1A) and 293T cells (Fig. 1B).

Unlike HB-EGF, Betacellulin (BTC) shedding is mediated by ADAM10 (Sahin et al., 2004) and ionomycin-induced calcium influx can stimulate ADAM10-dependent shedding (Sanderson et al., 2005; Horiuchi et al., 2007). Therefore, we

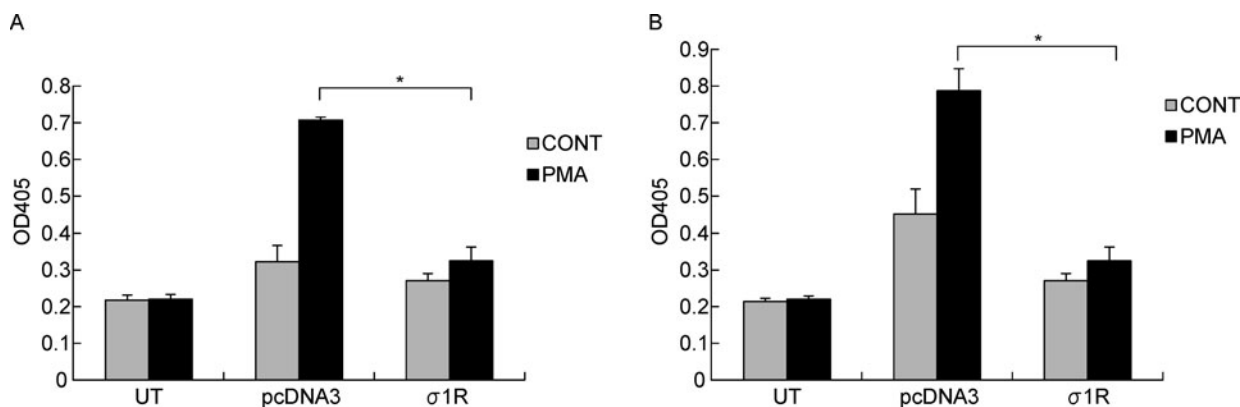


Figure 1. Overexpression of sigma-1 receptor could diminish ADAM17-dependent HB-EGF shedding *in vitro* on COS-7 cells (A) and 293T cells (B). Cells were transfected with proper plasmids and subjected to AP shedding assay. PMA (25 ng/mL) induced HB-EGF shedding was significantly reduced ($* p < 0.05$) when sigma-1 receptor was overexpressed. UT, untransfected cells; pcDNA3, cells transfected with pAPtag-HB-EGF and pcDNA3; σ 1R, cells transfected with pAPtag-HB-EGF and pcDNA3-flag-sigma1R.

used AP-tagged BTC to test the effect of sigma-1 receptor on ADAM10-dependent shedding. COS-7 or 293T cells were co-transfected with pAPtag5-BTC and pcDNA3-flag-sigma1R expressing constructs or with pAPtag5-HB-EGF and control pcDNA3 constructs. 24 h after transfection, cells were subjected to shedding assay and the supernatants were collected for further colorimetric assay. As shown in Fig. 2, 2.5 $\mu\text{mol/L}$ ionomycin-induced ADAM10-dependent BTC shedding was greatly inhibited when sigma-1 receptor was over-expressed in both COS-7 cells (Fig. 2A) and 293T cells (Fig. 2B).

Cholesterol modification and sigma-1 receptor agonist DHEAS could reduce ionomycin-induced ADAM10-dependent BTC shedding but not ADAM17-dependent HB-EGF shedding

Cholesterol is a ligand for sigma-1 receptor (Palmer et al., 2007) and its depletion can affect lipid raft formation and both ADAM10-dependent shedding (Murai et al., 2011) and ADAM17-dependent shedding (von Tresckow et al., 2004; Zimina et al., 2005; Tellier et al., 2006, 2008). Therefore, we tested the effect of cholesterol in our system. When cells were incubated with 1 mmol/L M β CD (a cholesterol depletion reagent) together with 2.5 $\mu\text{mol/L}$ ionomycin, the ionomycin-induced ADAM10-dependent BTC shedding was almost completely abolished (Fig. 3A; from 7.5-fold to 1.6-fold), while PMA induced ADAM17-dependent HB-EGF shedding was not affected at all (Fig. 3B). In fact, the ADAM10-dependent BTC shedding is very sensitive to membrane lipid modification as additional 5 $\mu\text{g/mL}$ cholesterol incubation could also largely reduce its shedding (Fig. 3A; from 7.5-fold to 3.8-fold). Interestingly, when 100 $\mu\text{mol/L}$ DHEAS, a natural

agonist of sigma-1 receptor (Maurice and Su, 2009), was added during induced shedding period, the ionomycin-induced ADAM10-dependent BTC shedding was also reduced from about 7.5-fold to 2.7-fold (Fig. 3A). It is likely that as a neurosteroid derived from cholesterol, DHEAS could compete with cholesterol and bind to a similar site on sigma-1 receptor and cause similar inhibition effect. On the other hand, the PMA induced ADAM17-dependent HB-EGF shedding was not sensitive to any lipid modification or the sigma-1 receptor agonist under the same condition (Fig. 3B).

Overexpressing sigma-1 receptor inhibited the sensitivity of ADAM10-dependent BTC shedding to lipid modification

Although the ADAM10-dependent BTC shedding was very sensitive to lipid modification and DHEAS treatment on COS-7 cells (Fig. 3A), such sensitivity was lost after overexpressing sigma-1 receptor (Fig. 4A). Overexpressing sigma-1 receptor could inhibit the ionomycin-induced ADAM10-dependent BTC shedding, and no further effect was observed when cells were co-incubated with either 1 mmol/L M β CD, or 5 $\mu\text{g/mL}$ cholesterol, or 100 $\mu\text{mol/L}$ DHEAS (Fig. 4A). Such effect is not due to any transcription or translation modification by overexpressing sigma-1 receptor as the expression level of ADAM10 was similar (Fig. 4B). Therefore, the effect might be due to the molecular chaperone function of sigma-1 receptor which could affect ADAM10 protein transportation on the membrane system. On the other hand, although the PMA induced ADAM17-dependent HB-EGF shedding was still not sensitive to any of those three drugs (Fig. 4C), the constitutive HB-EGF shedding was also inhibited by overexpressing sigma-1 receptor (Fig. 4C, indicated by # $p < 0.05$).

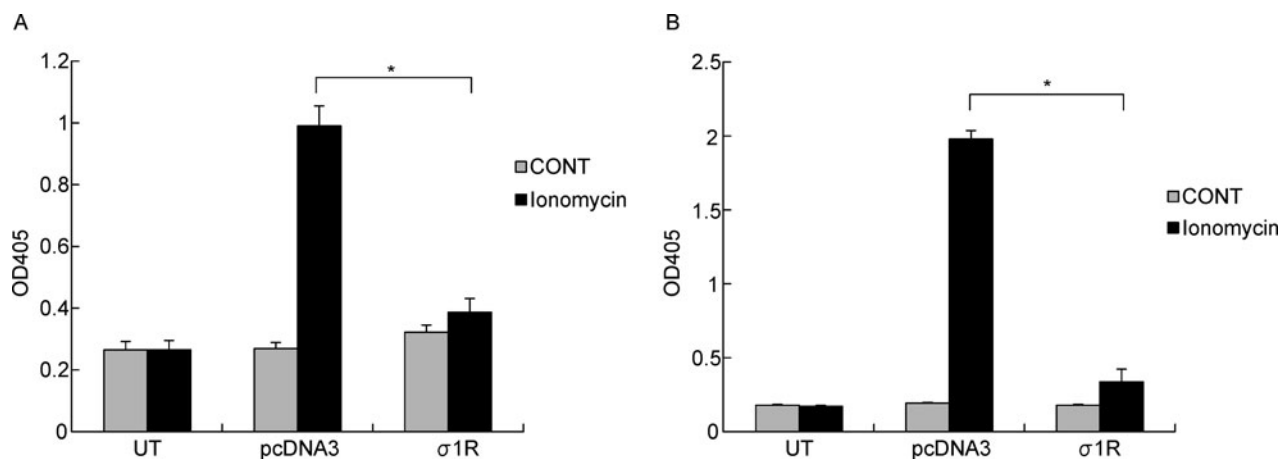


Figure 2. Overexpression of sigma-1 receptor could diminish ADAM10-dependent BTC shedding *in vitro* on COS-7 cells (A) and 293T cells (B). Cells were transfected with proper plasmids and subjected to AP shedding assay. Ionomycin (2.5 $\mu\text{mol/L}$) induced BTC shedding was significantly reduced (* $p < 0.05$) when sigma-1 receptor was overexpressed. UT, untransfected cells; pcDNA3, cells transfected with pAPtag-BTC and pcDNA3; σ 1R, cells transfected with pAPtag-BTC and pcDNA3-flag-sigma1R.

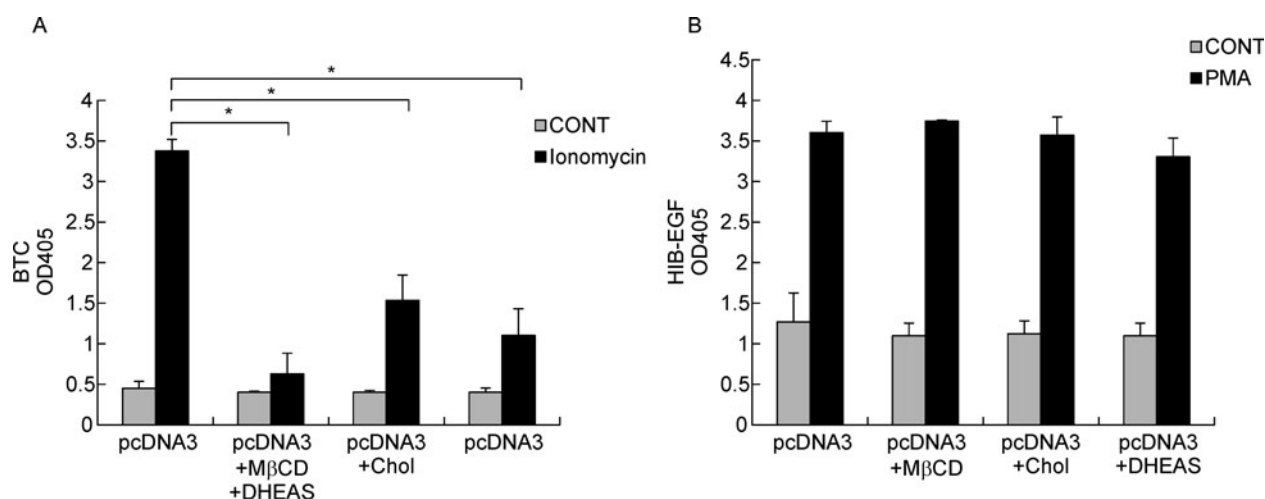


Figure 3. ADAM10-dependent BTC shedding is more sensitive to membrane lipid change while ADAM17-dependent HB-EGF shedding was not. (A) 2.5 $\mu\text{mol/L}$ ionomycin-induced ADAM10-dependent BTC shedding was very sensitive to lipid modification on COS-7 cells. 1 mmol/L M β CD could severely decrease ADAM10-dependent BTC shedding from 7.5-fold in control group to 1.6-fold. 5 $\mu\text{g/mL}$ cholesterol could reduce ADAM10-dependent BTC shedding to 3.8-fold and 100 $\mu\text{mol/L}$ DHEAS both could reduce ADAM10-dependent BTC shedding to 2.7-fold ($*p < 0.05$). (B) 25 ng/mL PMA induced ADAM17-dependent HB-EGF shedding was not sensitive to lipid modification or DHEAS treatment on COS-7 cells.

DISCUSSION

Sigma-1 receptors have been reported to be able to remodel lipid raft and affect many cellular functions. In our study, we used AP tagged substrates to test the effect of sigma-1 receptors on two sheddases, ADAM17 and ADAM10. Our results showed that over-expression of sigma-1 receptors could severely reduce both ADAM17-dependent HB-EGF shedding and ADAM10-dependent BTC shedding (Fig. 1 and 2). Both metalloproteases have been indicated to be involved in various diseases, such as brain pathology, inflammation, and cancer (Mochizuki and Okada, 2007; Pruessmeyer and Ludwig, 2009). The inhibition effect of sigma-1 receptor on those two enzymes suggested that it could be involved in pathogenesis of those diseases as well.

Interestingly, we also observed that the two enzymes showed different sensitivity to lipid modification (Fig. 3) even though they shared many substrates (Pruessmeyer and Ludwig, 2009). Such a difference could be due to either different distribution on cellular membrane or different activation mechanisms of those two enzymes. For ADAM10-dependent BTC shedding, cholesterol balance on membrane seems to be very important as both cholesterol depletion and addition could inhibit this shedding process (Fig. 3A). One possibility is that similar to ADAM10's function as α -secretase for APP, ADAM10 is located and function in the nonraft region (Harris et al., 2009) and its function is inhibited in the cholesterol-rich raft environment (Kojro et al., 2010). As cholesterol depletion with M β CD can increase the amount of sigma-1 receptor in lipid raft fractions in breast

cancer cell lines (Palmer et al., 2007), it is very likely that cholesterol depletion also increased the amount of sigma-1 receptor in lipid raft fraction in our experiments and such translocation might cause relocation of ADAM10 and in turn inhibit its function. On the other hand, ADAM17 has been reported to locate at both lipid raft region (Thiel and Carpenter, 2006; Takaguri et al., 2011) and nonraft region (Parr-Sturgess et al., 2010). Therefore, it is likely ADAM17 could shed HB-EGF in both microenvironments and therefore cholesterol depletion had no effect on ADAM17-dependent HB-EGF shedding (Fig. 3B). The detailed mechanism will need further investigation.

Alternatively, the different activation mechanisms of those two enzymes could also contribute to their different sensitivity to those drugs. Our results showed that additional cholesterol and DHEAS could also inhibit ADAM10-dependent BTC shedding (Fig. 3A). Such an effect might be due to the fact that those neurosteroids are sigma-1 receptor agonists (Maurice et al., 2006; Dong et al., 2009; Hayashi and Su, 2010) and activation of the sigma-1 receptor resulted in a bipolar modulation of calcium homeostasis in cell and high concentration of agonist could inhibit the free Ca^{2+} in cell (Hayashi et al., 2000; Maurice et al., 2006). Since ADAM10 function is induced by free Ca^{2+} in cell (Sanderson et al., 2005; Horiuchi et al., 2007), such inhibition could thus inhibit ADAM10-dependent BTC shedding. On the other hand, although sigma-1 receptor agonists could activate PKC (Fu et al., 2010; Yoon et al., 2010) which can in turn activate ADAM17-dependent shedding (Zheng et al., 2002, 2004; Sahin et al., 2004), such an effect might be covered by the

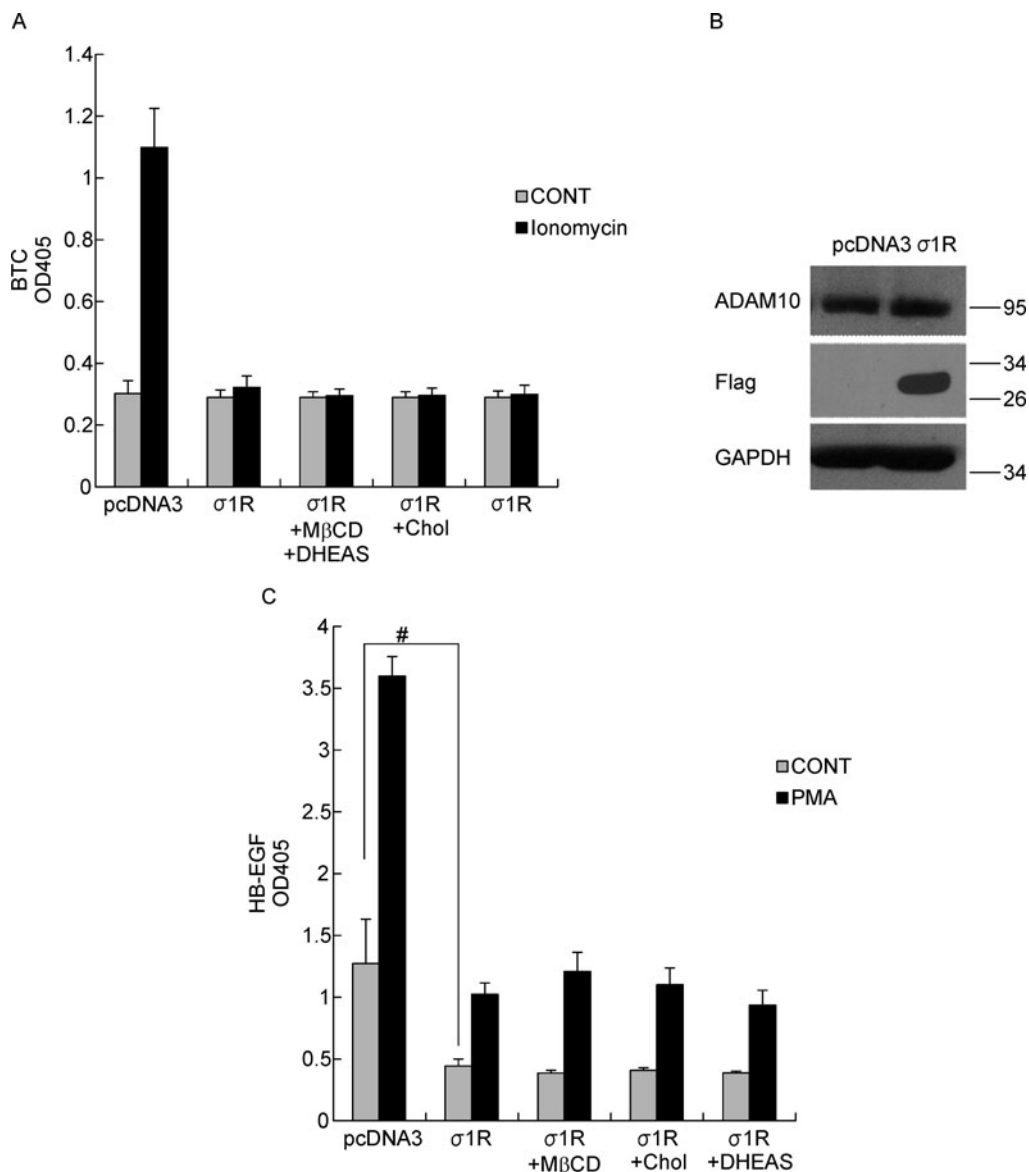


Figure 4. Overexpressing sigma-1 receptor inhibits the sensitivity of ADAM10-dependent BTC shedding to lipid modification. (A) The ADAM10-dependent BTC shedding was abolished by overexpressing sigma-1 receptor and further lipid modification or DHEAS treatment could not change the effect of overexpressing sigma-1 receptor. (B) Western blot showed that the expression level of ADAM10 was not changed after overexpressing sigma-1 receptor. (C) ADAM17-dependent HB-EGF shedding was inhibited by overexpressing sigma-1 receptor and still not sensitive to either lipid modification or DHEAS treatment. In addition to the PMA induced HB-EGF shedding, the constitutive shedding was also significantly inhibited by overexpressing sigma-1 receptor (# $p < 0.05$).

maximal PKC induction by PMA.

In conclusion, we demonstrated that overexpressing sigma-1 receptor could largely inhibit both ADAM10- and ADAM17-dependent shedding. The ionomycin-induced ADAM10-dependent BTC shedding was very sensitive to lipid cholesterol modification and the natural sigma-1 receptor agonist DHEAS could also inhibit ADAM10-dependent BTC shedding. Such inhibition indicates that sigma-1 receptor

could very likely be involved in some diseases by regulating ADAM10/17 function.

MATERIALS AND METHODS

Constructs and reagents

Sigma-1 receptor was cloned into pcDNA3-flag construct between

*Bam*HI and *Eco*RI sites with sense primer: 5'-CGGGATCCATG-CAGTGGGCCGTGGGCCGGC-3' and antisense primer: 5'-GGAATTC AAGGGTCCTGGCCAAAGAGG-3' to generate the flag-sigma1R expressing construct. BTC was cloned into pAPtag5 vector between *Xho*I and *Xba*I sites with sense primer: 5'-CTCTCGAG-GATGGGAACACAACC-3' and antisense primer: 5'-TTCTAGA-GAAGCAATATTGGTCTC-3' to generate the pAP-BTC construct. HB-EGF was cloned into pAPtag5 vector between *Xho*I and *Xba*I sites with sense primer: 5'-CTCTCGAGGAGAGTCTGGAGCGG-3' and antisense primer: 5'-TTCTAGAGAGTG GGAGCTAGCCAT-3' to generate the pAP-HB-EGF construct. M β CD, cholesterol, and DHEAS (dehydroepiandrosterone sulfate) were purchased from Sigma. PMA (phorbol 12-myristate 13-acetate) and ionomycin were purchased from Beyond.

Cell culture, transfection, and Western blot

COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, and 1% penicillin/streptomycin and co-transfected with mouse sigma-1 receptor expression plasmid pcDNA3-flag-sigma1R and pAPtag-HB-EGF constructs with Fugene6 (Roche) according to the manufacturer's protocol. After 24 h, transfected cells were washed with PBS, and then lysed in 300 μ L of cell lysis buffer (PBS, pH 7.4, with 1% (v/v) TritonX-100 and 5 mmol/L 1,10-phenanthroline) per well. Lysates were cleared by centrifugation at 13,000 rpm for 30 min. The supernatants were subjected to Western blot. In brief, protein samples mixed with sample loading buffer were loaded and separated by SDS-PAGE and then electrically blotted to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories). After overnight incubation in blocking buffer (5% non-fat dry milk, 10 mmol/L Tris-HCl pH 7.5, NaCl, KCl, 0.1% Tween20) at 4°C, immunoblots were incubated with proper antibody (dilution in TBST buffer: 10 mmol/L Tris-HCl pH 7.5, NaCl, KCl, 0.1% Tween20) for 16 h at 4°C followed by secondary antibody for 2 h at room temperature. Blots were then washed and incubated with enhanced chemiluminescence (ECL, Millipore) and detected by Kodak film.

AP shedding assay & Statics

The alkaline phosphatase tagged substrate shedding assay has been described previously (Zheng et al., 2002, 2004; Sahin et al., 2004). In brief, cells seeded in six-well tissue culture plates (Corning) were transfected with the appropriate expression plasmids using Fugene6 reagent (Roche). To measure shedding of the introduced AP-fusion proteins under basal conditions, cells were washed once with PBS, and then cultured in OptiMem for 1 h. The OptiMem medium was collected and replaced with fresh OptiMem medium with 25 ng/mL phorbol 12-myristate 13-acetate (PMA), or ionomycin (2.5 μ mol/L) at the indicated concentration for another hour to assess shedding under stimulated or inhibited conditions. All supernatants were collected and subjected to colorimetric assay with 4-NPP (4-nitrophenylphosphate, AMRESCO) as substrate of alkaline phosphatase and the readout was measured at 405 nm by Thermo Fluoroskan Ascent ELISA reader. All experiments were repeated at least three times and data were analyzed by T-test. Differences were taken as statistically significant at $p < 0.05$.

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ABBREVIATIONS

ADAM17, a disintegrin and metalloprotease 17; ADAM10, a disintegrin and metalloprotease 10; AP, alkaline phosphatase; DHEAS, dehydroepiandrosterone; M β CD, methyl-beta-cyclodextrin; σ 1R, sigma-1 receptor, sigma1R

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