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Cholesterol depletion induces ANTXR2-dependent activation of MMP-2 via ERK1/2 phosphorylation in neuroglioma U251 cell

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ABSTRACT

Cholesterol is a critical component of lipid rafts implicated in regulating multiple signal transduction. The anthrax toxin receptor 2 (ANTXR2) is a type I membrane protein acting as the second receptor for the anthrax toxin. In this study, we first investigated the association between cholesterol and ANTXR2. We provided the evidence that cholesterol depletion by methyl-beta-cyclodextrin (MβCD) promoted ANTXR2 expression in U251 neuroglioma cell, which was reversed by cholesterol supplement. MβCD-induced ANTXR2 up-regulation contributed to ERK1/2 phosphorylation, which was responsible for MT1-MMP and MMP-2 activation. Our data suggested that cellular cholesterol regulated ANTXR2-dependent activation of MMP-2 via ERK1/2 phosphorylation in neuroglioma U251 cell.

1. Introduction

Eukaryotic cellular membranes contain abundant cholesterol that plays a key role in maintaining structural integrity and receptor function [1]. Cholesterol and sphingolipids are first assembled into lipid rafts in the Golgi, and are subsequently moved towards the plasma membrane [2]. Lipid rafts are dynamic microdomains enriching in many functional molecules, including epidermal growth factor receptor (EGFR), transforming growth factor β receptor (TGF-βR), Fas and integrins [3]. These molecules have been demonstrated to regulate cell proliferation, apoptosis, migration, invasion and chemoresistance [4]. Cholesterol depletion disrupts lipid rafts and causes aberrant signal transductions [5]. For example, methyl-beta-cyclodextrin (MβCD), a cholesterol depletion agent, induces the up-regulation of EGFR and HIF-α in Human epidermoid carcinoma A431 cell [6], and promotes Fas activation in human keratinocyte HaCaT cell [7]. These results indicate that cellular cholesterol is critical for integrity of lipid rafts and proper signal transduction.

The anthrax toxin receptor 2 (ANTXR2) is a cell surface protein serving as the second receptor for the anthrax toxin, also known as capillary morphogenesis gene 2 (CMG2) [8]. The transmembrane receptor is closely related to angiogenic processes including endothelial cell proliferation and tube formation, which is due in part to interaction with extracellular matrix (ECM) proteins [9]. ANTXR2 has been implicated in regulation of ECM homeostasis because of its protein structure and binding capability [10]. ANTXR2 is abundantly expressed in murine tissues and is required for mouse parturition [11]. ANTXR2 is also highly expressed in cultured human umbilical venous endothelial cells (HUVEC) and breast tumor basement membranes, tumor stroma and microvessels, but is not discovered in breast tumor cells [8]. Furthermore, there have been little reports about ANTXR2 expression in other tumor cells. However, EST data from NCBI suggest that ANTXR2 expression is distributed in different tissue types [12].

Given that cholesterol depletion influences the expression of membrane proteins, in the present study, we investigated the effect of cholesterol depletion on ANTXR2 expression in neuroglioma U251 cell. We provided the evidence that cellular cholesterol depletion promoted ANTXR2 expression, which was responsible for ERK phosphorylation and activation of MMP-2.

2. Materials and methods

2.1. Antibodies and reagents

The monoclonal antibodies (mAbs) and polyclonal antibodies (pAbs) used in this study containing: anti-ANTXR2 (pAb) and anti-phospho-ERK1/2 (pAb) were purchased from Abcam (Cambridge, MA). Anti-beta-actin (pAb) was from Anbo Biotechnology Company (San Francisco, CA, USA). Anti-MT1-MMP was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). RIPA lysis buffer was

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purchased from Cell Signaling Technology (Beverly, MA). Methyl-beta-cyclodextrin (MβCD), water-soluble cholesterol, U0126, PMA and gelatin were purchased from Sigma Chemical Co. (USA).

2.2. Cell culture

The human neuroglioma cell line U251 were purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone, USA) supplemented with 10% fetal bovine serum (Hyclone, Australian), 100 U/mL penicillin, and 100 U/mL streptomycin at 37 °C in 5% CO_2.

2.3. Cholesterol assay

For cholesterol depletion experiments, U251 cells were incubated in serum-free DMEM medium for 4 h and treated with varying concentrations of MβCD for 1 h. Then, the cells were washed with PBS and incubated in DMEM medium for 1 h. For cholesterol recovery experiments, after MβCD treatment, the cells were washed with PBS and further incubated with 1 mM cholesterol for 1 h. Free cholesterol concentration was measured by Free Cholesterol Assay Kits (Applygen Technologies, Beijing, China). Each experiment was performed in triplicate.

2.4. ANTXR2 gene silencing

The control siRNA and ANTXR2 siRNA oligos were synthesized by GenePharma (Shanghai, China). The ANTXR2 target sense sequence was 5′-CCTGCACCTATCCTGAATAAA-3′ [13], and the negative control siRNA sense sequence was 5′-TTCTCCGAACGTGTCACGT-3′ [14]. Cell transfection was performed according to manufacturer's instructions. Briefly, U251 cells were incubated at 2×10^5 cells/well in a 6-well plate. After grown to approximately 70% confluence, cells were transfected with ANTXR2 siRNA or control siRNA using Lipofectamine 2000 (Invitrogen).

2.5. Western blot analysis

The treated cells were lysed in RIPA lysis buffer and supernatants were separated by centrifugation at 14000 rpm for 20 min at 4 °C. The concentration of solubilized protein was quantified using the bicinchoninic acid method. Equal amounts of proteins were separated using 10% SDS–PAGE. Subsequently, the proteins were electrotransferred onto polyvinylidene fluoride membranes. After blocked with 5% BSA for 1 h at room temperature, the membranes were probed with anti-ANTXR2 (1:1000), anti-phospho-ERK1/2 (1:2000), anti-MMP-2 (1:1000) and anti-actin (1:2000) antibodies overnight at 4 °C. The membranes were washed three times with TBST and then, incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The labeled proteins were detected using the enhanced chemiluminescence method and quantified using Alpha Imager2200.

2.6. Gelatin zymography

Gelatin zymography analysis was performed as described previously [15]. Briefly, after treatment, the conditional media were collected (1.0 ml per culture condition) and were concentrated by ultrafiltration (Millipore). The samples were dissolved in Laemmli buffer without DTT under nondenaturating conditions. An aliquot (40 μl) of samples were subjected to 8% SDS–PAGE containing 1 mg/ml gelatin. After electrophoresis, the gels were incubated in 2.5% Triton X-100 for 60 min at 37 °C and rinsed three times in double-distilled H_2O. The gel was further incubated in the developing buffer (50 mm Tris–HCl, 20 mm NaCl, 5 mm $CaCl_2$, 0.02% Brij 35, pH 7.5) at 37 °C for 24 h, then stained with 0.5% Coomassie Brilliant Blue overnight and destained with destaining buffer (30% methanol, 10% acetic acid in H_2O). Proteinolytic activity was determined as the clear zones under the blue background.

2.7. Flow cytometry analysis

Flow cytometric analysis of ANTXR2 expression on the surface of cells was performed as described previously [16]. Briefly, cells were harvested by brief trypsinization and incubated with 1:100 dilution of anti-ANTXR2 antibody. After rinsed three times with PBS, cells were further incubated with 1:1000 dilution of TRITC-conjugated goat anti-rabbit antibody. The labeled cells were analyzed by a flow cytometer (Beckman Coulter Corp.) and the data were processed using WinMDI 2.9 software.

2.8. Immunofluorescence

Cells grown on cover slips were fixed with 4% paraformaldehyde for 15 min, then permeabilized with 0.2% Triton X-100 for 5 min and blocked with 3% BSA for 1 h at room temperature. After rinsed three times with PBST, cells were incubated with 1:100 dilution of anti-ANTXR2 antibody overnight at 4 °C, followed by incubation with FITC-conjugated secondary antibody IgG for 1 h. After rinsed five times with PBST, The labeled cells were detected using a fluorescence microscope (LEICA DMIRE2).

2.9. Statistical analysis

Statistical analysis was performed using the SPSS software (SPSS 18.0). The data were representative of at least three independent experiments. The comparisons between two groups were evaluated by Student's *t*-test. The comparisons among multiple groups were assessed by one-way ANOVA. Probability values of less than 0.05 were considered to be statistically significant.

3. Results

ANTXR2 is a membrane protein and plays a critical role in early life stage. ANTXR2 is necessary for murine parturition [10,11], and defects in ANTXR2 are the cause of infantile systemic hyalinosis (ISH) and juvenile hyaline fibromatosis (JHF) [10]. It has been proposed that ANTXR2 protein is important for maintaining the structure of basement membranes [9]. However, little was understood about the relation between ANTXR2 and cellular cholesterol.

3.1. Cellular cholesterol regulated ANTXR2 expression

To investigate whether cellular cholesterol mediated ANTXR2 expression, cholesterol depletion and repletion assays were performed. As shown in Fig. 1A, cholesterol depletion by 0.8 mM MβCD for 1 h promoted ANTXR2 expression, which could be attenuated by adding 1 mM cholesterol. The data from Flow cytometric analysis further confirmed these results (Fig. 1B), suggesting that cellular cholesterol regulated ANTXR2 expression.

3.2. Cellular cholesterol regulated ANTXR2 expression in dose-dependent manner

To further test the influence of cellular cholesterol level on ANTXR2 expression, we treated U251 cells with varying concentrations of MβCD. MβCD induced cholesterol depletion in dose-dependent manner (Fig. 2). As increasing dose, MβCD decreased gradually cellular cholesterol level. Exposure to 0.8 mM MβCD for 1 h reduced

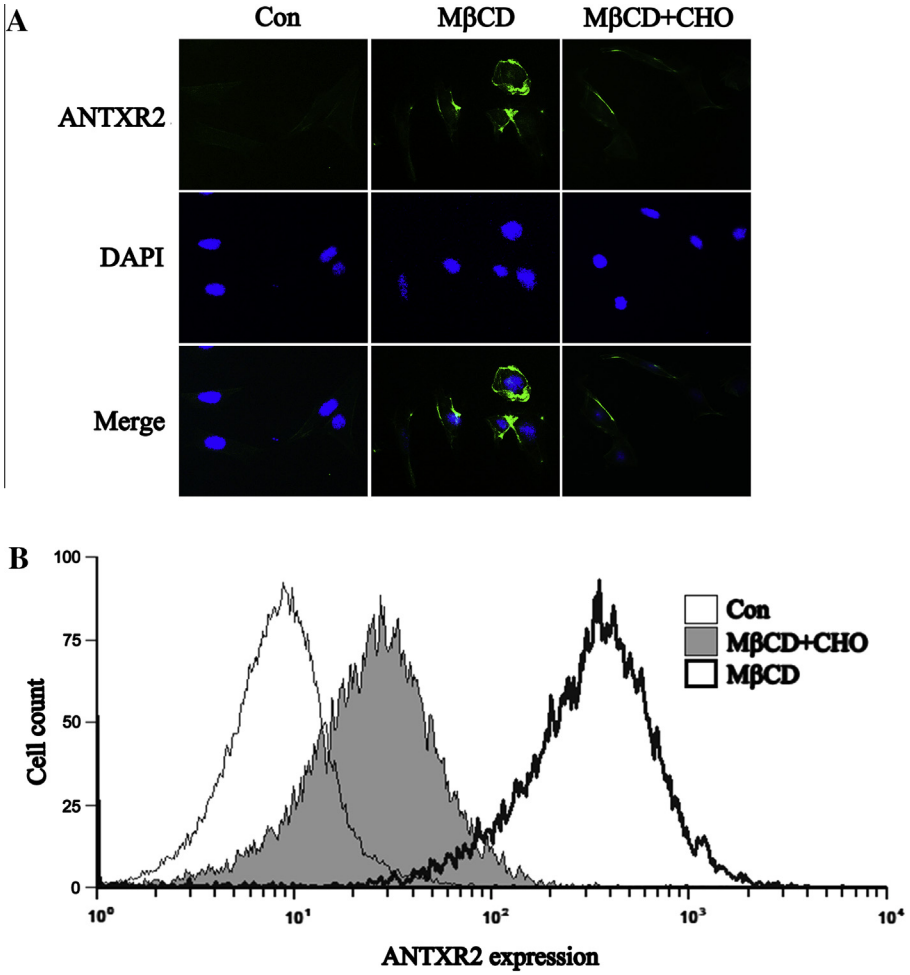

Fig. 1. MβCD promoted ANTXR2 expression in neuroglioma U251 cell. U251 cells were incubated in serum-free DMEM medium for 4 h and subsequently treated with or without 0.8 mM MβCD for 1 h, followed by incubation with 1 mM water-soluble cholesterol or medium only for 1 h. (A) Detection of ANTXR2 expression on U251 cells surface by immunofluorescence. (B) Analysis of ANTXR2 expression on U251 cells surface by flow cytometry.

cholesterol level by approximately 50%. We further studied the influence of cholesterol depletion on ANTXR2 expression. Western blot analysis showed that MβCD dose dependently promoted ANTXR2 expression (Fig. 2). As cholesterol level was reduced, ANTXR2 expression was gradually enhanced. In contrast, after MβCD treatment, cholesterol supplement increased significantly cellular cholesterol content, and attenuated ANTXR2 expression. These results indicated that cellular cholesterol regulated ANTXR2 expression in dose-dependent manner.

3.3. Cellular cholesterol regulated ERK phosphorylation in dose-dependent manner

To assessed the influence of cellular cholesterol on ERK phosphorylation, U251 cells were treated with MβCD at different doses for 1 h. The results showed that MβCD significantly promoted ERK phosphorylation at the doses of 0.4, 0.8 and 1.6 mM, and cholesterol supplement attenuated ERK phosphorylation induced by MβCD.

3.4. Cellular cholesterol regulated activation of MT1-MMP and MMP-2

Membrane type-1 matrix metalloproteinase (MT1-MMP) acts as a proteolytic enzyme contributing to degradation of extracellular matrix and tumor invasion. It has been demonstrated that cholesterol depletion in human fibrosarcoma cells (HT1080) induces accumulation of MT1-MMP [17]. We further investigated the effect of cellular cholesterol level on MT1-MMP activation in U251 cells. Our results indicated that cholesterol depletion induced activation of MT1-MMP in dose-dependent manner while replenishing cholesterol in cholesterol-depleted cells reversed MT1-MMP activation. Because MT1-MMP has been identified as the physiological activator of MMP-2 [18], we also studied the influence of cellular cholesterol content on MMP-2 activation determined by gelatin zymography. The results suggested that cholesterol depletion promoted the accumulation of active MMP-2 but, had no effects on MMP-9. Cholesterol supplement in cholesterol-depleted cells also reversed MMP-2 activation but, had no effects on MMP-9.

3.5. ANTXR2 regulated MT1-MMP and MMP-2 activation via ERK phosphorylation

We further tested whether ANTXR2 regulated ERK phosphorylation and activation of MT1-MMP and MMP-2. As shown in Fig. 3A, ANTXR2 knockdown by siRNA interference reduced ERK phosphorylation level and inhibited activation of MT1-MMP and MMP-2. In contrast, MβCD treatment promoted ANTXR2 expression and elevated ERK phosphorylation level. As expected, MβCD treatment also activated MT1-MMP and MMP-2. These results indicated that ANTXR2 was indeed implicated in mediating ERK phosphorylation and activation of MT1-MMP and MMP-2. Subsequently, we investigated that whether blocking ERK

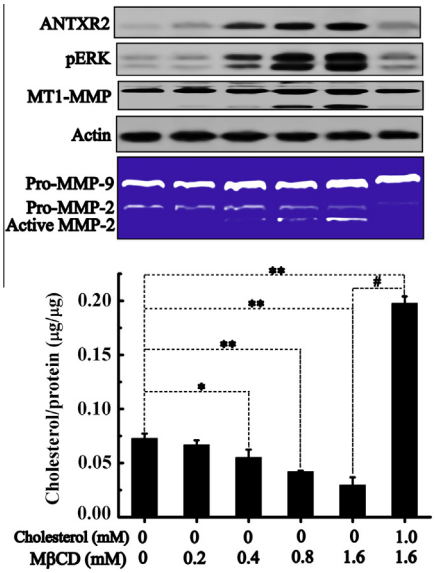

Fig. 2. Cholesterol content regulated ANTXR2 expression, ERK phosphorylation, activation of MT1-MMP and MMP-2. U251 cells were incubated in serum-free DMEM medium for 4 h and subsequently treated with or without varying concentrations of MβCD for 1 h, followed by incubation with 1 mM water-soluble cholesterol or medium only for 1 h. Western blot analysis was used to detect the ANTXR2 expression, ERK phosphorylation and MT1-MMP activation. Gelatin zymography analysis was used to detect MMP-2 activation. Free cholesterol concentration was measured by Free Cholesterol Assay Kits. $^{*}p < 0.05$ vs. control group; $^{**}p < 0.01$ vs. control group; $^{\#}p < 0.01$ vs. the group treated with 1.6 mM MβCD.

phosphorylation influenced activation of MT1-MMP and MMP-2. U0126, a known ERK inhibitor, is usually used to inhibit ERK phosphorylation. The results showed that U0126 completely blocked ERK phosphorylation, even in the presence of MβCD (Fig. 3B). Blocking ERK phosphorylation abolished MβCD-induced activation of MT1-MMP and MMP-2 (Fig. 3B). PMA, a strong activator of ERK, was used to promote ERK phosphorylation in this study. As shown in Fig. 3B, PMA significantly elevated ERK phosphorylation level and promoted activation of MT1-MMP and MMP-2. However, neither U0126 nor PMA had any effects on ANTXR2 expression. These results indicated that ANTXR2 regulated MT1-MMP and MMP-2 activation via ERK phosphorylation.

4. Discussion

In this study, we demonstrated that cellular cholesterol was implicated in mediating ANTXR2 expression. Cholesterol is a critical component of lipid raft locating on plasma membrane. Thus, cellular cholesterol regulates the expression of various membrane proteins, such as caveolin-1, integrins and EGFR [18]. ANTXR2 is a transmembrane protein involved in the homeostasis of the extracellular matrix (ECM) [10], and it shares similar characteristics with integrins [9]. However, there were little reports about the association between cholesterol and ANTXR2. Furthermore, the molecular roles of ANTXR2 remains unknown. This report demonstrated that MβCD, a cholesterol depletion agent, induced up-regulation of ANTXR2 in dose-dependent manner, and replenishing cholesterol in cholesterol-depleted cells reversed MβCD-induced events. These results suggested that cellular cholesterol content regulated ANTXR2 expression. It has been well known that MβCD causes the caveolae internalization rendering cells more accessible to the ECM [17]. ANTXR2 has been implicated in mediating ECM Homeostasis [10], which is likely to explain the influence of cholesterol depletion on ANTXR2 expression.

Membrane type-1 matrix metalloproteinase (MT1-MMP) is the first member of MMP family and acts as the activator of pro-MMP-2 [19]. MT1-MMP is a functional protein degrading ECM components and directly regulates ECM turnover [10]. Hence, it is necessary to investigate whether ANTXR2 mediates MT1-MMP activation. Our results revealed that depletion of cholesterol by MβCD promoted ANTXR2 expression and MT1-MMP activation in parallel. Consistent with previous reports, cholesterol depletion causes the accumulation of active MT1-MMP by inhibiting

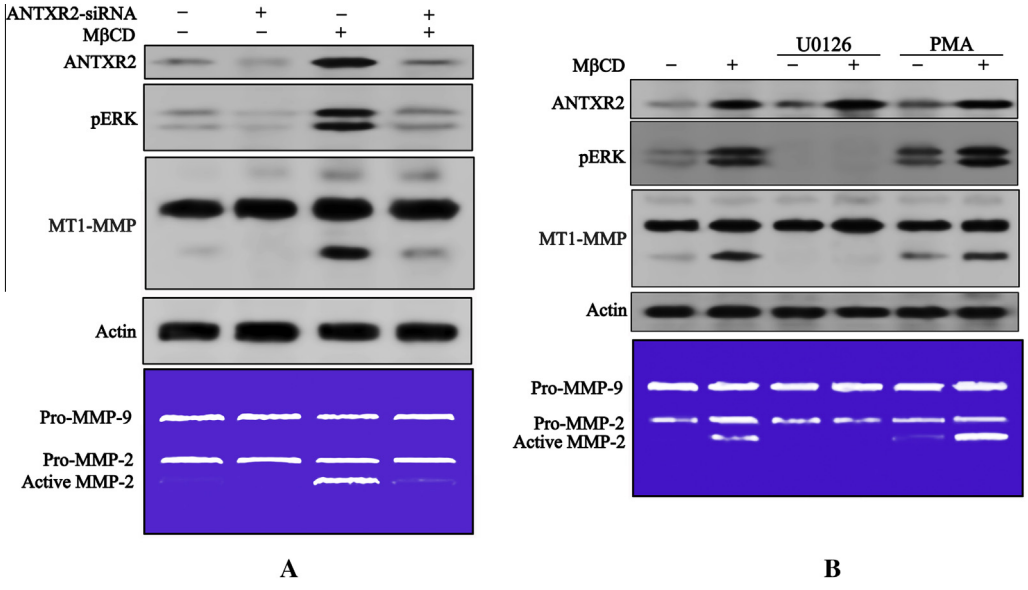

Fig. 3. MβCD induced ANTXR2-dependent MMP-2 activation via ERK phosphorylation. (A) U251 cells were transfected with ANTXR2 siRNA or negative control siRNA using Lipofectamine 2000 for 12 h. Subsequently, media were replaced with serum-free DMEM media, and cells were further cultured for 4 h followed by treatment with or without 0.8 mM MβCD for 1 h. Western blot analysis was used to detect the ANTXR2 expression, ERK phosphorylation and MT1-MMP activation. Gelatin zymography analysis was used to detect MMP-2 activation. (B) Serum-starved U251 cells were pre-incubated with vehicle control (DMSO) or 10 μM U0126 or 100 nM PMA for 4 h. Then, media were replaced with or without 0.8 mM MβCD for 1 h. Western blot analysis was used to detect the ANTXR2 expression, ERK phosphorylation and MT1-MMP activation. Gelatin zymography analysis was used to detect MMP-2 activation.

endocytosis [17,20]. However, little reports about the relation between ANTXR2 and MT1-MMP have been published. This study demonstrated that MT1-MMP activation was inhibited by transfection of ANTXR2 siRNA, suggesting that ANTXR2 regulated MT1-MMP activation. ANTXR2 siRNA also suppressed MMP-2 activation. Because MT1-MMP regulates MMP-2 activation, this result indicated that ANTXR2 conducted MT1-MMP-mediated MMP-2 activation.

Following the above observations, the question how ANTXR2 regulates MT1-MMP is raised. Previous reports have demonstrated that MβCD induced ERK phosphorylation in diverse cell types [21–23]. Our results demonstrated that, accompanied with up-regulation of ANTXR2, ERK phosphorylation level was enhanced by MβCD in U251 cells. We further tested whether ERK phosphorylation was involved in ANTXR2-mediated MT1-MMP activation. ANTXR2 siRNA abolished ERK phosphorylation induced by MβCD, but neither inhibitor or activator of ERK have any effects on ANTXR2 expression. These results indicated that ANTXR2 should be an up-stream modulator of ERK. Interestingly, inhibiting ERK significantly decreased MT1-MMP and MMP-2 activation induced by MβCD. Combined with the result that ERK activator stimulated MT1-MMP and MMP-2 activation, it was concluded that ERK mediated MT1-MMP and MMP-2 activation. Consistent with these results, Mou et al. have also suggested that ERK contributes to the activation of MT1-MMP and MMP-2 in U251cells [24].

In summary, we provided the evidences that cholesterol depletion by MβCD promoted ANTXR2 expression in U251 neuroglioma cell. ANTXR2 up-regulation further induced ERK phosphorylation. ERK phosphorylation was responsible for MT1-MMP and MMP-2 activation. MβCD-induced signal transduction could be reversed by cholesterol supplement.

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