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TNF-α induces expression of urokinase-type plasminogen activator and β-catenin activation through generation of ROS in human breast epithelial cells

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ABSTRACT

Malignant tumors have a capability to degrade the extracellular matrix (ECM) by controlled proteolysis. One of the important components of the proteolysis system involved in such process is urokinase-type plasminogen activator (uPA). Tumor necrosis factor (TNF)-α was found to stimulate uPA. TNF-α impaired the ability of cells to aggregate and to attain compaction. Dyscohesion (cell–cell dissociation) induced by TNF-α was associated with the disordered expression of cadherin/β-catenin at the sites of cell–cell contact. We observed that human breast epithelial (MCF-10A) cells treated with TNF-α transiently up-regulated expression of uPA and its mRNA transcript. In addition, TNF-α induced activation of β-catenin in MCF-10A cells. Based on these findings, we attempted to examine the role of β-catenin and its partner, Tcf-4 in upregulation of uPA. siRNA knock down of β-catenin abrogated TNF-α-induced uPA expression as well as Tcf-4/β-catenin DNA binding. TNF-α-stimulated MCF-10A cells exhibited increased intracellular accumulation of reactive oxygen species (ROS). TNF-α-induced expression of uPA and activation of β-catenin signaling appear to be mediated by ROS in MCF-10A cells, as both events were blocked by the antioxidant N-acetylcysteine.

Eupatilin (5,7-dihydroxy-3′,4′,6-tri-methoxy-flavone), a pharmacologically active flavone derived from *Artemisia asiatica*, has been shown to possess strong antioxidative activity. Eupatilin inhibited TNF-α-induced intracellular ROS accumulation, expression of uPA and β-catenin activation. Moreover, eupatilin inhibited the TNF-α-induced invasion of MCF-10A cells. Taken together, the above results suggest that eupatilin has chemopreventive effects on mammary tumorigenesis by targeting the β-catenin-uPA axis stimulated by TNF-α.

1. Introduction

Metastasis, one of the most detrimental causes associated with malignant progression, is a complex cascade of events involving tumor dissemination from the primary site of growth to distant organs. Metastasis is multistep events involving degradation or rearrangement of the extracellular matrix (ECM), local invasion, angiogenesis, intravasation, survival of malignant cells during the circulation, extravasation and the growth at a secondary site [1]. One of the key mediators causally involved in the aforementioned processes is the serine protease, urokinase-type plasminogen activator (uPA) [2]. Members of the plasminogen activator system, including urokinase plasminogen activated receptor (uPAR), have been found to be over-expressed in a large number of tumors, particularly breast cancer, which is associated with a poor prognosis [2,3]. uPA catalyzes the formation of plasmin from plasminogen to generate the proteolytic cascade that contributes to the breakdown of ECM, a key step in cancer metastasis [4,5].

During the metastatic cascade, many pathogenic changes occur. These include inflammation mediated by over-secretion of cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-8 [6,7]. TNF-α is a pleiotropic pro-inflammatory cytokine that has a wide range of biological activities. TNF-α can mediate tumor progression by inducing the cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis [8–10]. Many of TNF-α-induced cellular responses and alterations have been associated with accumulation of reactive oxygen species

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(ROS) [11,12]. ROS are relatively harmless, but when produced excessively or in the absence of sufficient antioxidant defense, the balance between oxidants and antioxidants is disturbed. This may lead to the initiation and promotion of cancer [13–15]. ROS produced by TNF-α activates several intracellular signaling molecules, such as β-catenin and its partner T-cell factor (Tcf)-4 [16,17]. β-Catenin has been shown to have a dual role as a major structural component of cell–cell adherence junctions and also as a transcription activator. In addition, β-catenin is involved in tumorigenesis by transactivating the lymphoid enhancer factor/T-cell factor (Lef/Tcf) transcription factor. Some of the genes whose expression is up-regulated by β-catenin/Tcf signaling are *c-jun*, *c-myc*, *fibronectin*, *cyclin D1*, *mmp* and *uPA* [18,19].

In the present study, we found that treatment of immortalized human breast epithelial (MCF-10A) cells with TNF-α resulted in the increased expression of uPA with concurrent enhancement of invasive capacity. Interestingly, TNF-α-induced uPA up-regulation and cell invasion were mediated by ROS that triggered the activation of β-catenin signaling. As TNF-α is a typical pro-inflammatory cytokine, its overactivation of β-catenin signaling may contribute to the inflammation-associated carcinogenesis. In this context, it is noticeable that β-catenin-mediated signaling has recently been recognized as an important molecular target for chemoprevention with anti-inflammatory substances [19]. *Artemisia asiatica* (Asteracease), widespread in nature, has been frequently used in traditional oriental medicine for the treatment of inflammation, cancer, and microbial infections [20–22]. Our previous studies have shown that eupatilin (5,7-dihydoxy-3′,4′,6-tri-methoxy-flavone), one of the pharmacologically active ingredients of *A. asiatica*, possesses anti-inflammatory and anti-tumor promoting activities [21]. This prompted us to examine the ability of this flavonoid to inhibit TNF-α-induced expression of uPA, to activate β-catenin and to cause invasion of MCF-10A cells.

2. Materials and methods

2.1. Materials

Euaptilin was supplied from Dong-A Pharmaceutical Co. Ltd. (Yong-In Si, South Korea) and dissolved in DMSO for treatment. TNF-α, sodium dodecylsulfate (SDS), N-acetylcysteine (NAC), cholera toxin, hydrocortisone, insulin and human recombinant epidermal growth factor (h-EGF) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM)/Ham's nutrient mixture F-12 (1:1) and horse serum were obtained from Gibco BRL (Grand Island, NY, USA). Dichlorofluorescein diacetate (DCF-DA) was purchased from Molecular Probes, Inc. (Eugene, OR, USA). Primers for *uPA* and *glyceraldehydes-3-phosphate dehydrogenase* (*GAPDH*) were synthesized by Bionics (Seoul, South Korea). Negative control and β-catenin short interfering RNA (siRNA) were purchased from Ambion (Austin, TX, USA).

2.2. Cell culture

MCF-10A cells were kindly supplied by Prof. Aree Moon (Duksung Women's University, South Korea). The cells were cultured in DMEM/F-12 medium supplemented with 5% heat-inactivated horse serum, 10 μg/ml insulin, 100 ng/ml cholera toxin, 0.5 μg/ml hydrocortisone, 20 ng/ml h-EGF, 2 mmol/l L-glutamine and 100 units/ml penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2/95% air.

2.3. Western blot analysis

MCF-10A cells were lysed in RIPA lysis buffer [150 mM NaCl, 0.5% Triton X100, 50 mM Tris–HCl (pH 7.4), 25 mM NaF, 20 mM EGTA, 1 mM dithiothreitol (DTT), 1 mM Na_3VO_4, protease inhibitor cocktail tablets] for 15 min on ice followed by centrifugation at 12,000 × *g*

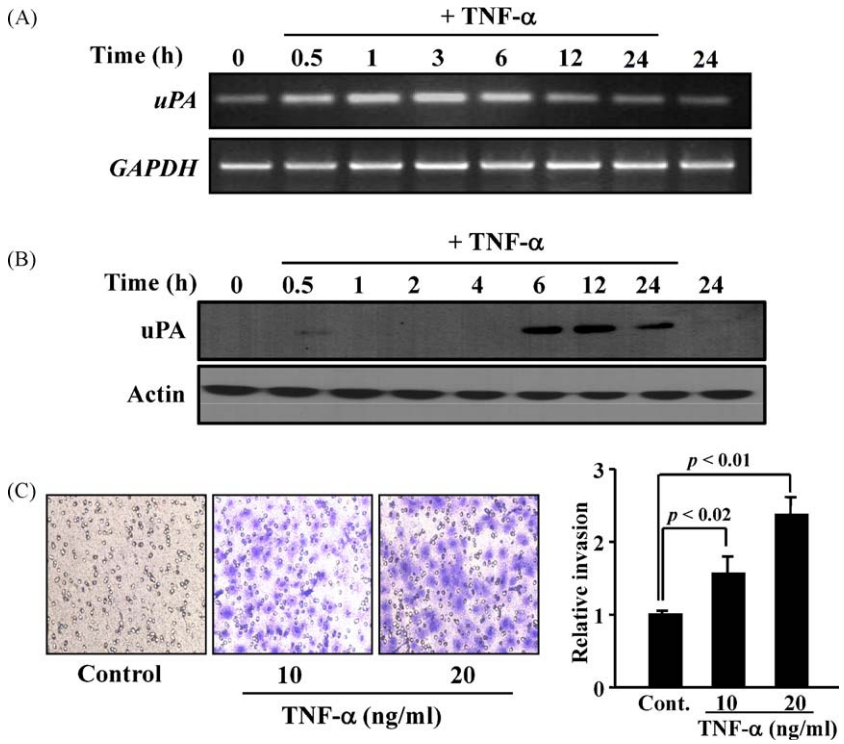

Fig. 1. TNF-α-induced invasiveness and uPA expression in MCF-10A cells. (A) The expression of uPA mRNA was determined by semi-quantitative RT-PCR at various time intervals after treatment with 10 ng/ml TNF-α. (B) TNF-α-induced uPA protein expression was assessed by Western blot analysis using anti-uPA antibody after treatment with 10 ng/ml TNF-α for indicated times. (C) After treatment of MCF-10A cells with TNF-α for 24 h, an *in vitro* invasion assay was performed as describe in Section 2.

for 20 min. The protein concentration of the supernatant was measured by using the BCA reagents (Pierce, Rockfold, IL, USA). Protein (30 μg) was separated by running through 8–12% SDS-PAGE gel and transferred to the PVDF membrane (Gelman Laboratory, Ann Arbor, MI, USA). The blots were blocked with 5% non-fat dry milk-PBST buffer [PBS containing 0.1% Tween-20] for 1 h at room temperature. The membranes were incubated for 2 h at room temperature with 1:1000 dilution of each antibody. Antibody against uPA was purchased from Calbiochem (Cat. No. IM 15L; Cambridge, MA, USA) and β-catenin antibody was obtained from Santacruz Biotechnology (Cat. No. sc-65481; Santa Cruz, CA, USA). Equal lane loading was assured using actin (Sigma Chemical Co., St. Louis, MO, USA). The blots were rinsed three times with PBST buffer for 5 min each. Washed blots were treated with 1:5000 dilution of the horseradish peroxidase conjugated-secondary antibody (Zymed Laboratories, San Francisco, CA, USA) for 1 h and washed again three times with PBST buffer. The transferred proteins were visualized with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

2.4. Measurement of intracellular accumulation of ROS

To monitor the intracellular accumulation of ROS, the fluorescent probe DCF-DA was used. Following treatment, cells were rinsed with Kreb's ringer solution and 25 μM DCF-DA was loaded. After 15-min incubation at 37 °C, cells were examined under a confocal microscope (Leica Microsystems Heidelberg GmbH, Heidelberg, Germany) equipped with an argon laser (488 nm, 200 mW).

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from MCF-10A cells using Trizol® reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. One microgram of total RNA was reverse transcribed with murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) at 42 °C for 50 min and 72 °C for 15 min. PCR conditions for *uPA* and for the house keeping gene, *glyceraldehyde-3-phosphate dehydrogenate* (*GAPDH*) are as follows: for *uPA*, 26 cycles for 94 °C for 30 s, 59 °C for 40 s, and 72 °C for 50 s; *GAPDH*, 26 cycles for 94 °C for 1 min, 56 °C for 2 min, and 72 °C for 2 min. The primer pairs and the size of the expected products are as follows (forward and reverse, respectively): uPA, 5′-CGGGGGGGCTCTGTCACCTAC-3′ and 5′-CGGCCCCAGCTCACAATTCC-3′, 594 bp; *GAPDH*, 5′-AAGGTCGGAGTCAACGGATTT-3′ and 5′-GCAGTGAGGGTCTCTCTCTTCCT-3′, 1053 bp. Amplication products were resolved by 1–1.2% agarose gel electrophoresis, stained with ethidium bromide, and photographed under ultraviolet light.

2.6. Electrophoretic mobility shift assay (EMSA)

Confluent cells in 100 mm dishes were treated with TNF-α. Cells were gently washed twice with ice-cold phosphate-buffered saline (PBS), scraped in 1 ml PBS and centrifuged at 12,000 × *g* for 30 s at 4 °C. Pellets were suspended in 200 μl of hypotonic buffer A (10 mM HEPES, pH 7.9; 10 mM KCl; 2 mM $MgCl_2$; 1 mM DTT; 0.1 mM EDTA; 0.1 mM PMSF) for 15 min on ice, and 12.5 μl of 10% Non-diet P-40 solution was added for 5 min. The mixture was then centrifuged for 6 min at 12,000 × *g*, and the pellets were washed once with 400 μl of PBS, suspended in 70 μl of buffer C (50 mM HEPES, pH 7.9; 50 mM KCl; 300 mM NaCl; 0.1 mM EDTA; 1 mM DTT; 0.1 mM PMSF; 10% glycerol) for 20 min on ice and centrifuged for 6 min at 12,000 × *g*. The supernatant containing nuclear proteins was collected and stored at −70 °C after determination of the protein concentration. EMSA was performed using a DNA-protein binding detection kit according to the manufacturer's protocol (GIBCO, Grand Island, NY, USA). Briefly, the Tcf-4 oligonucleotide probe (5′-CCC TTT GAT CTT ACC-3′) was labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase and separated from free [γ-^{32}P]ATP by gel filtration using a nick spin column (Amersham Biosciences, UK). Prior to addition of the ^{32}P-oligonucletide (100,000 cpm), 10 μg of nuclear extract was kept on ice for 15 min in gel shift binding buffer [10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 4% (v/v) glycerol, and 0.1 mg/ml sonicated salmon sperm DNA]. To determine the sequence specificity of the Tcf-4 DNA interaction, the excess amount of unlabeled oligonucleotides was added. After 20-min incubation at room temperature, 2 μl of 0.1% bromophenol blue was added, and samples were electrophoresed through 6% non-denaturing polyacrylamide gel at 150 V in a cold room. Finally, the gel was dried and exposed to an X-ray film.

2.7. In vitro invasion assay

An *in vitro* invasion assay was performed using a 24-well Transwell unit with polycarbonate filters, 6.5 mm in diameter with a pore size of 8.0 μm (Corning Costar, Cambridge, MA, USA). The lower side of the filter was coated with 10 μl of 0.5 mg/ml type I collagen, and the upper side was coated with 10 μl of 0.5 mg/ml reconstituted basement membrane substance (Matrigel; Collaborative Research, Lexington, KY, USA). The coated filters were air-dried for 1 h before

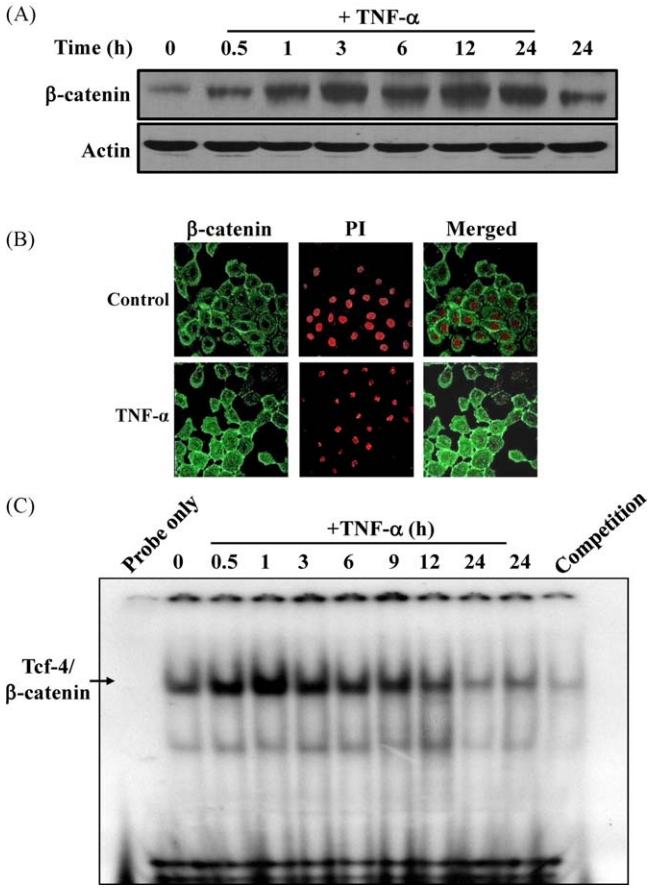

Fig. 2. TNF-α-induced activation of β-catenin in MCF-10A cells. (A) TNF-α-induced β-catenin expression in whole cell lysates was examined by Western blot analysis using anti-β-catenin antibody after treatment with 10 ng/ml TNF-α for indicated time periods. (B) Immunocytochemical analysis was performed using anti-β-catenin antibody after the treatment of MCF-10A cells with TNF-α. Cells were stained with propidium iodide (PI) and analyzed by confocal microscopy. (C) TNF-α-induced β-catenin-Tcf-4 DNA binding in MCF-10A cells. TNF-α-induced β-catenin-Tcf-4 DNA binding activity was assessed by EMSA with nuclear extracts prepared at indicated time intervals after treatment with 10 ng/ml TNF-α.

the assay. The lower compartment was filled with 600 μl of media containing 0.1% bovine serum albumin (BSA). Fifty thousand cells were resuspended in 100 μl of media, and placed in the upper part of the Transwell plate. Cells were incubated for 24 h in a humidified atmosphere of 5% CO_2 at 37 °C. Cells were fixed with methanol and stained with hematoxylin and eosin. Cells on the upper surface of the filter were removed mechanically by wiping with a cotton swab, and the invasive phenotypes were determined by counting cells that migrated to the lower side of the filter with microscopy at 200×. For better quantification of invasion, invaded cells on the bottom side of the membrane were stained with 0.1% crystal violet solution, and then cells on the upper surface of the filter were removed by cotton swab. The relative invasion was determined by counting the number of cells in thirteen fields that invaded the Matrigel matrix relative to the number of cells that migrated through the control insert.

2.8. Transient transfection

MCF-10A cells were seeded at a density of 2×10^5/well in a 6-well dish and grown to 60–70% confluence in complete growth media. For each well, pre-validated β-catenin siRNA (Ambion, Austin, TX, USA) was transfected into cells using lipofectamine

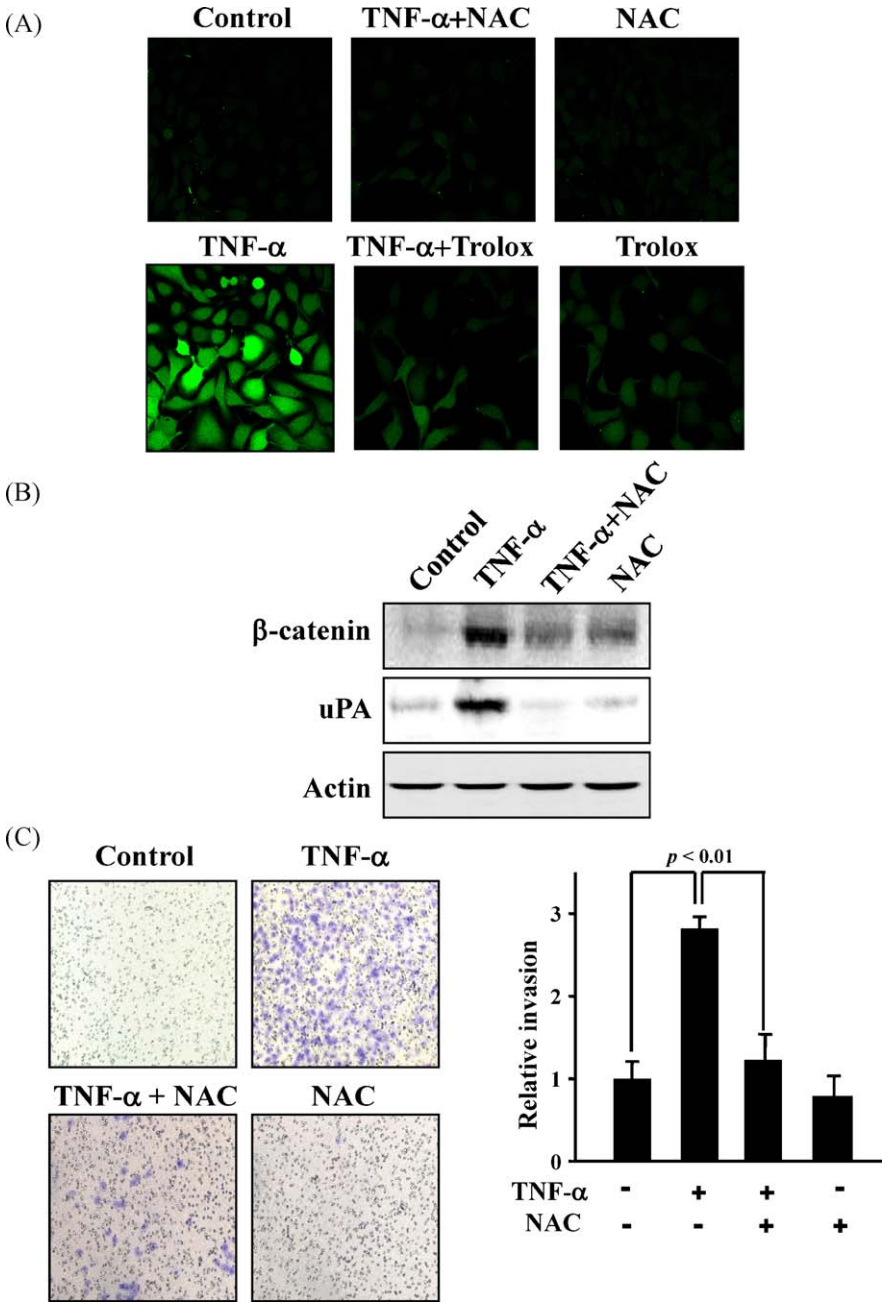

Fig. 3. TNF-α-induced intracellular accumulation of ROS in MCF-10A cells. (A) Intracellular ROS levels were determined based on the DCF-DA fluorescence. Cells were exposed to 10 ng/ml TNF-α alone or in combination with NAC (5 mM) or Trolox (50 μM) for 1 h. Images of the cellular fluorescence were acquired by using a confocal laser-scanning microscope. (B) Effects of NAC on TNF-α-induced expression of β-catenin and uPA. Cells were exposed to 10 ng/ml TNF-α alone or in combination with NAC (5 mM) for 12 h, and levels of β-catenin and uPA were examined by Western blot analysis. (C) After treatment of MCF-10A cells with TNF-α in the presence or absence of NAC for 24 h, an *in vitro* invasion assay was performed as describe in Section 2. The left panel illustrates invaded cells stained with hematoxylin and eosine, while the right panel represents the relative invasion assessed by staining the cells with crystals violet in separate experiments.

RNAiMAX reagents (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.9. Immunofluorescence staining

MCF-10A cells grown on four-well chamber slides were exposed to 10 ng/ml TNF-α for 3 h. Cells were rapidly rinsed with phosphate-buffered saline (PBS), and fixed for 10 min at 4 °C with 4% formaldehyde. After washed with PBS, the cells were blocked by PBS containing 5% BSA and 0.5% Tween-20 for 1 h at room temperature. The translocation of β-catenin was visualized using a rabbit polyclonal antibody (sc-7199; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The antibody was diluted 1:100 in blocking buffer and incubated overnight at 4 °C. Afterwards, cells were washed with PBS and labeled with FITC-conjugated goat anti-rabbit IgG (Zymed Laboratories, Inc., San Francisco, CA, USA) diluted 1:1000 for 1 h at room temperature. Cells were rinsed with PBS, and stained cells were analyzed under a confocal microscope and photographed (Leica Microsystems Heidelberg GmbH, Heidelberg, Germany).

3. Results

3.1. TNF-α induced expression of uPA and invasion in MCF-10A cells

When human breast epithelial (MCF-10A) cells were stimulated with TNF-α (10 ng/ml), mRNA (Fig. 1A) and protein (Fig. 1B) levels of uPA were elevated in a time-dependent manner. In addition, the invasive capacity of MCF-10A cells was significantly increased by TNF-α treatment for 24 h (Fig. 1C), suggesting that the upregulation of uPA expression is essential for development of the invasiveness in TNF-α treated MCF-10A cells.

3.2. TNF-α-induced activation of β-catenin in MCF-10A cells

In another experiment, we examined whether TNF-α could induce β-catenin expression by Western blot analysis using anti-β-catenin antibody after treatment with TNF-α (10 ng/ml). We noted that treatment of MCF-10A cells with TNF-α resulted in the elevated β-catenin protein expression in a time-related manner (Fig. 2A). When activated by Wnt signaling, β-catenin translocates to the nucleus where it forms a transcriptional complex with TCF/lymphoid enhancer factor family proteins, such as TCF-4 [23]. To elucidate the nuclear translocation of β-catenin, we examined its subcellular distribution by the immunocytochemistry. As illustrated in Fig. 2B, MCF-10A cells treated with TNF-α exhibited increased levels of β-catenin accumulation in the nucleus. In addition, TNF-α induced the DNA binding of β-catenin-Tcf-4 in MCF-10A cells (Fig. 2C).

3.3. TNF-α-induced β-catenin activation and invasiveness of MCF-10A cells were mediated by ROS

Considering that TNF-α induces intracellular ROS generation in many different cell types, we measured the ROS production in TNF-α stimulated MCF-10A cells. MCF-10A cells treated with TNF-α displayed intense fluorescence inside the cell upon staining with DCF-DA dye, indicative of ROS accumulation. As shown in Fig. 3A, the increased ROS accumulation in TNF-α treated MCF-10A cells was markedly reduced by antioxidants, NAC and trolox. In a subsequent experiment, TNF-α-induced expression of β-catenin and uPA was attenuated by NAC (Fig. 3B). Moreover, the invasiveness of MCF-10A treated with TNF-α was almost abolished by NAC treatment (Fig. 3C). These findings suggest the possible involvement of oxidative stress in TNF-α-induced MCF-10A cell invasion.

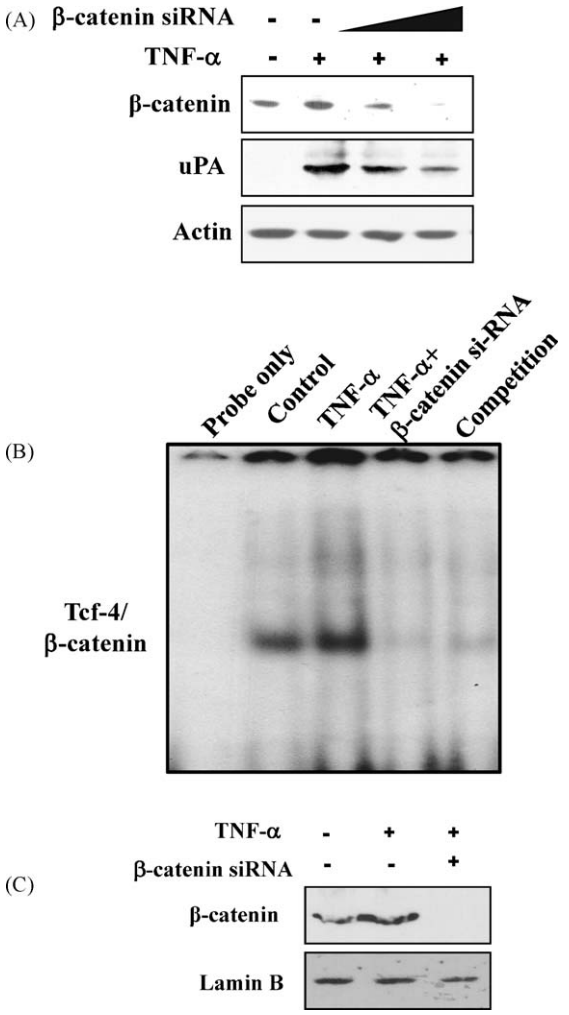

Fig. 4. TNF-α-induced expression of uPA mediated by β-catenin signaling. (A) MCF-10A cells were transiently transfected with β-catenin siRNA (10 or 25 nM) for 36 h by using RNAiMax reagent according to the manufacturer's instructions. Transfectant cells were treated with 10 ng/ml TNF-α for 12 h, and expression of β-catenin and uPA was examined by Western blot analysis using anti-β-catenin and uPA antibodies, respectively. (B) MCF-10A cells were transiently transfected with β-catenin siRNA (25 nM) for 24 h. Transfectant cells were treated with 10 ng/ml TNF-α for 1 h, and the β-catenin-Tcf-4 DNA binding activity was assessed by EMSA.

3.4. TNF-α-induced expression of uPA was mediated via β-catenin signaling

To elucidate the molecular mechanism underlying TNF-α-induced invasion, MCF-10A cells were transfected with the siRNA oligonucleotides to knock down the *β-catenin* gene expression. As a result, TNF-α-induced expression of uPA (Fig. 4A) and β-catenin-Tcf-4 DNA binding (Fig. 4B) were decreased following introduction of β-catenin siRNA into MCF-10A cells. These results suggest that TNF-α-induced expression of uPA is mediated via the β-catenin signaling.

3.5. Eupatilin inhibited TNF-α-induced ROS production, β-catenin activation, uPA expression and invasion

Pre-incubation of MCF-10A cells with the antioxidant flavone eupatilin significantly inhibited TNF-α-induced intracellular accumulation of ROS (Fig. 5A). Likewise, TNF-α-induced β-catenin accumulation and uPA expression were attenuated by eupatilin pre-treatment (Fig. 5B). In addition, treatment with eupatilin

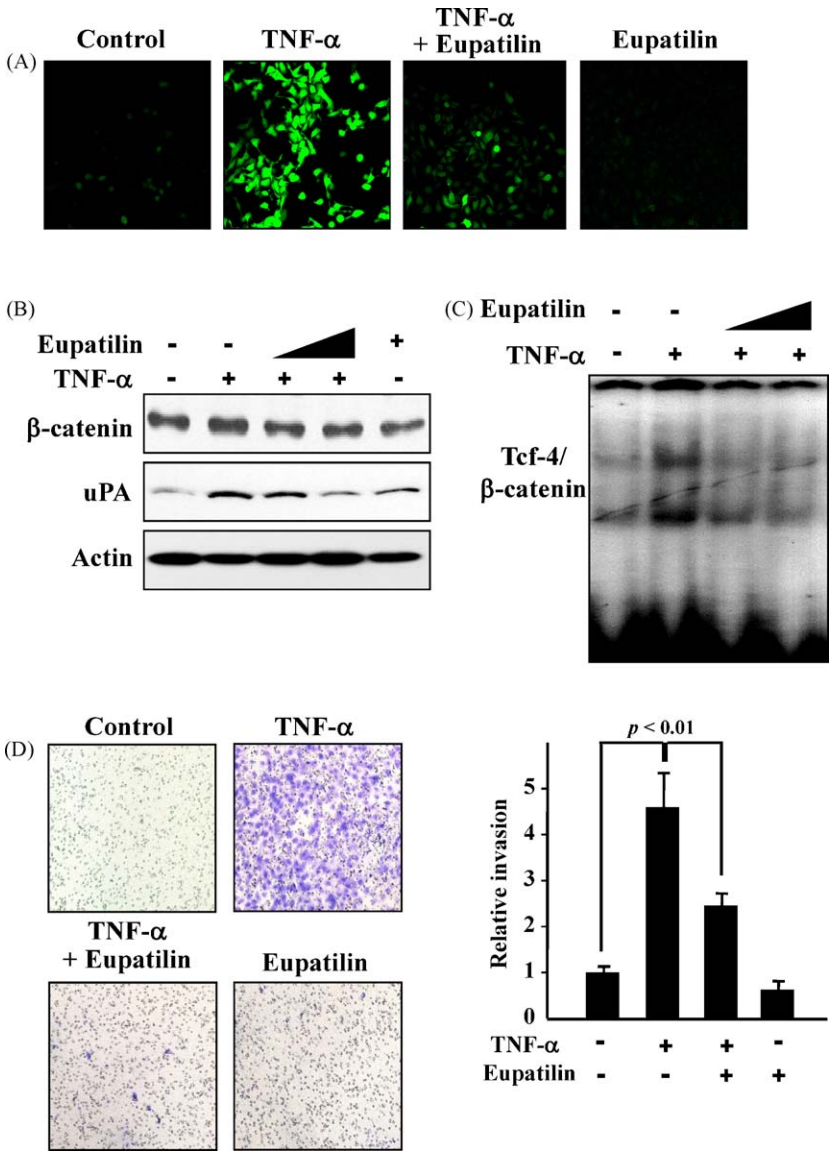

Fig. 5. Effects of eupatilin on TNF-α-induced intracellular accumulation of ROS and invasiveness in MCF-10A cells. (A) Intracellular ROS levels were determined based on the DCF-DA fluorescence. MCF-10A cells were pre-treatment with eupatilin (50 μM) for 1 h and exposed to 10 ng/ml TNF-α alone or in combination with eupatilin for 12 h. Images of the cellular fluorescence were acquired by using a confocal laser-scanning microscope. (B) Cells were exposed to 10 ng/ml TNF-α alone or in a combination with eupatilin (10 or 50 μM) for 12 h, and expression of β-catenin and uPA was examined by Western blot analysis. (C) Cells were exposed to 10 ng/ml TNF-α alone or in combination with eupatilin (10 or 50 μM) for 1 h, and β-catenin/Tcf-4 DNA binding activity was assessed by EMSA. (D) After treatment of MCF-10A cells with TNF-α in the presence or absence of eupatilin (50 μM) for 24 h, an *in vitro* invasion assay was performed as describe in Section 2. The left panel illustrates invaded cells stained with hematoxylin and eosine, while the right panel represents the relative invasion assessed by staining the cells with crystal violet in separate experiments.

inhibited the TNF-α-stimulated activation of β-catenin-Tcf-4 DNA binding (Fig. 5C). Moreover, the invasive capability of TNF-α-treated MCF-10A cells was attenuated by eupatilin treatment (Fig. 5D). Taken together, the above findings suggest that eupatilin inhibits the TNF-α-induced upregulation of uPA expression by blocking the activation of β-catenin signaling in human mammary epithelial cells, which would contribute to the suppression of the invasiveness of the cells.

4. Discussion

TNF-α is produced by tumors and can act as an endogenous tumor promoter. As a prototypic pro-inflammatory cytokine, TNF-α is considered as a molecular link between inflammation and cancer [9,24]. It also induces expression/production of other cytokines, angiogenic factors, MMPs, and uPA, contributing to increased growth and survival of tumor cells. Thus, Yin et al. have reported that TNF-α enhances the invasive capacity of human breast cancer (MCF-7) cells through upregulation of adhesion molecules and receptors including urokinase receptor [25]. In addition, there are reports demonstrating that TNF-α increases the expression and secretion of uPA in various normal cells, such as human ovarian surface epithelial (HOSE) cells [26], human dental pulp cells [27] and human keratinocytes (HaCaT) [28]. In agreement with above studies, our data reveal that TNF-α treatment induces the expression of uPA in human mammary epithelial (MCF-10A) cells.

The uPA converts inactive plasminogen into plasmin and hence plays an important role in the initiation of a cascade of proteolytic steps involved in the degradation of ECM [4,5]. The overexpression of uPA has been detected in breast cancer [29,30], and a high level of uPA in tumors is associated with a rapid disease progression and a poor prognosis [31,32]. Tumor invasion is mediated by ROS, angiogenic growth factors and their receptors and proteolytic

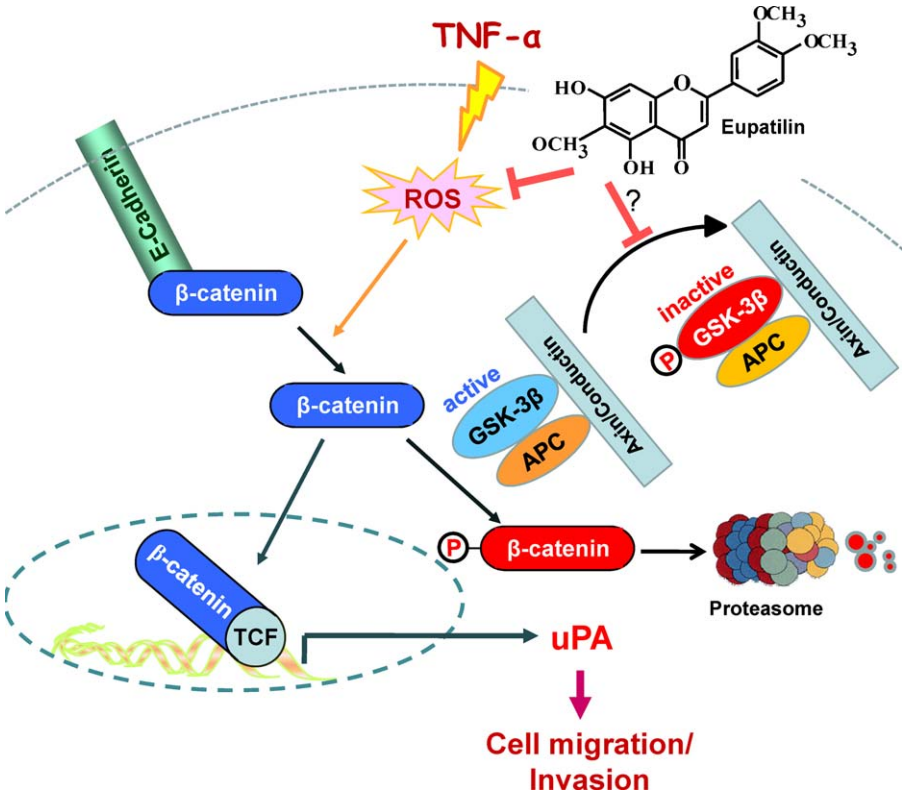

Fig. 6. A putative mechanism by which eupatilin suppresses TNF-α-induced invasiveness of MCF-10A cells. Eupatilin inhibits the TNF-α-induced upregulation of uPA expression and β-catenin activation by blocking the accumulation of ROS in MCF-10A human mammary epithelial cells, thereby attenuating the invasive potential of these cells induced by TNF-α. For details of ROS-mediated β-catenin accumulation/activation, refer to a reference [47]. Eupatilin may directly scavenge ROS or potentiate the *de novo* synthesis of antioxidant enzymes. Alternatively, eupatilin may inhibit the phosphorylation of GSK3β at the Ser-9 residue, thereby keeping this kinase in the catalytically active state. Activation of GSK3β by eupatilin facilitates β-catenin degradation, leading to suppression of β-catenin/TCF-mediated transcription of uPA and attenuation of migration and invasive potential of MCF-10A cells.

enzymes [33,34]. Kim et al. have reported that intracellular ROS accumulation precedes the induction of uPAR expression, and this upregulation is attenuated by NAC, a ROS scavenger [35]. In addition, exogenous ROS alone induced the expression and promoter activity of uPA [35,36]. Consistent with these findings, our present study also demonstrates that TNF-α increases the expression of uPA through ROS generation which was inhibited by the NAC treatment in MCF-10A cells. TNF-α-induced ROS production has been proposed as a plausible mechanism for inflammation-associated carcinogenesis [37].

Metastasis requires proteolytic degradation of ECM components to facilitate the invasion of malignant cells through the basement membrane and subsequently, the connective tissue [38]. uPA mediates extracellular signal transduction presumably through association with different types of integrins and with an ECM component [39,40]. Inappropriate activation of the β-catenin signaling by inflammatory mediators is implicated in carcinogenesis [41], and abnormally elevated levels of β-catenin have been associated with poor prognosis in breast cancer [42]. In addition, accumulating data from both *in vitro* and *in vivo* studies suggest the involvement of overactivated β-catenin-mediated signaling in tumorigenesis [19]. Nuclear localization of β-catenin and subsequent formation of the β-catenin-Tcf/LEF transcription complex cause enhanced transcription of uPAR [18,43]. This led us to examine association between expression of uPA, one of the putative target genes of β-catenin, and invasion in MCF-10A cells stimulated with TNF-α. Our results reveal that TNF-α mediates cell invasion by activating β-catenin signaling and subsequently upregulating uPA expression. Contrary to our findings, a recent study by Moreau et al. reveals that siRNA-mediated silencing of β-catenin increases expression of uPA, uPAR and plasminogen activator inhibitor-1 (PAI-1) in breast cancer (MCF-7 and MDA-MB-231) cells and also in colon cancer (SW-480) cells, thereby enhancing their invasive potential [44]. In the same study, treatment of β-catenin siRNA-transfected cells with SN50, a specific inhibitor of nuclear factor-kappaB (NF-κB), significantly reduced enhancement of uPA, uPAR and PAI-1 expression and cancer cell invasion. Furthermore, β-catenin siRNA-treated cells exhibited NF-κB nuclear accumulation. Based on these data, the authors have suggested that β-catenin down-regulates the uPA/uPAR system in cooperation with NF-κB [44]. The reason for such discrepancy between their study and ours is unknown, but may be due to different cell lines used.

β-catenin-mediated signaling, a principal hub in the inflammation signaling network, represents a critical molecular target of anti-inflammatory substances with chemopreventive or chemotherapeutic potential [19]. Tosetti et al. addressed that several signaling molecules of the inflammatory processes are involved in tumorigenesis and angiogenesis and that non-steroidal anti-inflammatory drugs (NSAIDs) have the anti-tumor activity when administered in the early stages of carcinogenesis [45]. Sulindac, a classical NSAID, suppresses β-catenin signaling pathways in lung cancer (A549), breast cancer (MCF-7), and colon cancer (SW620) cells [46]. In addition, a naturally occurring anti-inflammatory substance curcumin abrogated the cell proliferation through down-regulation of Wnt/β-catenin in human breast cancer (MCF-7 and MDA-MB-231) cells. Our study showed that eupatilin, an anti-inflammatory ingredient derived from *A. asiatica*, decreased TNF-α-induced uPA upregulation and β-catenin/Tcf-4 DNA binding activity in MCF-10A cells. The

compound also inhibited the invasiveness of MCF-10A cells induced by TNF-α.

According to current understanding of the Wnt/β-catenin signaling, β-catenin is targeted for degradation in the absence of growth signaling by the glycogen synthase kinase 3beta (GSK3β) that forms a β-catenin destruction complex with Axin and APC. The β-catenin signaling is normally turned on by Wnt proteins when they bind to a cell surface receptor, Frizzled. This leads to suppression of the phosphorylation of β-catenin by the GSK3β-APC-AxinA complex. Wnt/β-catenin signaling is subjected to redox regulation. Thioredoxin-related protein, nucleoredoxin (NRX) is considered to govern ROS-stimulated Wnt/β-catenin signaling [47]. NRX normally interacts with dishevelled (Dvl), an essential adaptor protein for Wnt signaling, and blocks the activation of Wnt/β-catenin signaling. Oxidative stress induced by ROS causes dissociation of NRX from Dvl, which enables Dvl to activate the β-catenin signaling [47] by inactivating GSK3β though serine-9 (Ser-9) phosphorylation. We speculate that the antioxidative flavone eupatilin, by attenuating the cellular ROS accumulation, may inhibit the dissociation of Dvl from NRX, renders GSK3β catalytically active, and facilitates phosphorylation and subsequent degradation of β-catenin, thereby blocking β-catenin-driven uPA expression (Fig. 6). However, the possibility that eupatilin potentiate the activity of GSK3β by directly blocking the phosphorylation of this kinase at the serine-9 residue cannot be excluded.

In summary, TNF-α overproduces ROS, which activate β-catenin. Activation of ROS-mediated β-catenin signaling and subsequent upregulation of uPA are likely to be responsible for the TNF-α-induced enhancement of invasive potential of MCF-10A cells and, therefore, could be implicated in the progression of breast carcinogenesis.

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