Andrographolide Inhibits PMA-Induced EPCR Shedding through JNK and p38 MAPK

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Abstract

Endothelial cell protein C (EPCR), one of the main members in the protein C (PC) pathway, plays an important role in the process of coagulation and inflammation. EPCR can shed from the cell membrane, which process is mediated by the tumor necrosis factor-α converting enzyme (TACE) and related to some diseases. Andrographolide is the major constituent of *Andrographis paniculata*, a kind of herbal medicine commonly used in clinical for its various pharmacological actions, especially anti-inflammation and anti-cancer. In this study, we investigated andrographolide protection for EPCR and its potential molecular mechanism. Phorbol-12-myristate-13-acetate (PMA)-stimulated human umbilical vein endothelial cells (HUVECs) were used to be the model of EPCR shedding and the andrographolide pretreatment was tested in this model. The results showed that andrographolide could reduce PMA-induced EPCR shedding and inhibit the TACE function. Additionally, we found andrographolide also suppressed the phosphorylation of c-Jun N-terminal kinase (JNK) and p38, but had no effect on extracellular regulated protein kinase (ERK) 1/2. Given these results, andrographolide can inhibit the shedding of EPCR by the suppression of TACE, which may take JNK and p38 as the molecular target. These findings

indicated a substantial anti-EPCR shedding efficacy of andrographolide in vitro.

Keywords : Andrographolide; EPCR; TACE; MAPK; HUVECs

Introduction

Endothelial protein C receptor (EPCR) was firstly identified as a novel type I transmembrane glycoprotein shown to be capacity of binding protein C (PC) (Fukudome and Esmon, 1994), and scientists found it was specifically expressed on endothelial cells, especially the large vessels (Fukudome et al. 1998). After that, EPCR was known to accelerate PC activation efficiently and bind with activated PC (APC) to indirectly involve in anticoagulant, anti-inflammation, anti-apoptosis and vascular barrier protection (Stearns-Kurosawa et al.1996; Esmon 2012). In addition, researchers believe that EPCR can be independent of APC to perform functions due to other ligands discovered in recent years (Pendurthi and Rao 2018). The membrane EPCR (mEPCR) existing on endothelial cells surface can be cleaved by metalloprotease and release in plasma as the soluble EPCR (sEPCR), which process is regulated by inflammatory mediators and thrombin (Xu et al. 2000). It is worthwhile to mention that sEPCR still has capacity for binding PC/APC, which can compete with mEPCR (Regan et al. 1996). sEPCR circulate in normal plasma with low concentration (Zheng et al. 2007). However, in some systemic inflammatory diseases (Sesin et al. 2005; Kurosawa et al. 1998; Gandrille 2008), supraphysiologic level of sEPCR can influence PC activation and APC activity (Kurosawa et al. 1997). Hence, EPCR shedding was considered to harm its

function (Esmon 2009).

Tumor necrosis factor-α converting enzyme (TACE), also named ADAM17, is an important member of the ADAM (a disintegrin and metalloproteinase) family (Zunke and Rose-John 2017). TACE participates many kinds of receptors shedding including the EPCR and shows complicated bioactivity (Qu et al. 2007). Phorbol esters belong to the tetranuclear diterpenoids generally known for potent tumor promoters (Goel et al. 2016), in which phorbol–12–myristate–13-acetate (PMA) is often used to stimulate human umbilical vein endothelial cells (HUVECs) EPCR shedding (Lee et al. 2016). PMA can exert effects by the phosphorylation of mitogen-activated protein kinases (MAPKs), such as extracellular regulated protein kinase (ERK) 1/2, p38 and c-Jun N-terminal kinase (JNK) (Shankar et al. 2016; Chao et al. 2016; Chen et al. 2019). Besides, the activation of MAPK signaling pathway has been proved to enhance TACE activity (Killock and Ivetic 2010; Menschikowski et al. 2009). Consequently, we assumed that PMA-induced sEPCR release may be regulated by MAPK pathway. And this hypothesis had been verified in our previous study (Ma et al. 2017).

Andrographis paniculata (*A. paniculata*) is a herbaceous plant, which has a long history of medicine and food application in Asia and Europe (Okhuarobo et al. 2014). As the primary compound separated from aerial part of *A. paniculata*, andrographolide has been reported that it possesses a variety of pharmacological effects like antibacterial, anti-inflammatory, antiviral, antioxidation, anticancer and so on (Zhang et al. 2018; Mussard et al. 2019; Islam 2017; Gupta et al. 2017; Dai et al. 2019). Moreover, its cardiovascular protective effect and corresponding potential therapeutic action have

drawn researchers wide attention (Maiti et al. 2006). Considering the vital role of EPCR in coagulation and inflammation, and there have never been a report about whether andrographolide has effect on EPCR shedding. In this study, we detected the ability of andrographolide for PMA-induced EPCR shedding in HUVECs, and tried to further explain relevant mechanism through MAPK signals.

Materials and methods

Reagents

Andrographolide ($C_{20}H_{30}O_5$, >98% purity) was obtained from herbpurify Co., Ltd. (Chengdu, China). Dimethyl sulfoxide (DMSO) and phorbol-12-myristate-13-acetate (PMA) were purchased from Sigma (St. Louis, MO, U.S.A.). Bisindolylmaleimide I (BIS) was purchased from CST (Beverly, MA, U.S.A.). PROCR (Human) enzyme-linked immunosorbent assay (ELISA) Kit was purchased from Abnova (Taipei, Taiwan). Antibody sources were as follows: human EPCR antibody (R&D Systems, Inc., Minneapolis, MN, U.S.A.); anti-ADAM17 antibody-Cytoplasmic domain (ab39162) (Abcam, Cambridge, U.K.); mitogen-activated protein kinase (MAPK) Family Antibody Sampler Kit#9926, Phospho-MAPK Family Antibody Sampler Kit#9910 (Cell Signaling Technology, Beverly, MA, U.S.A.); mouse monoclonal anti-beta-actin (sc-47778), goat anti-rabbit antibody, goat anti-mouse antibody, rabbit anti-goat antibody (Santa Cruz Biotech, Santa Cruz, CA, U.S.A.)

The preparation for reagents was as followed: Dissolve the PMA with cell cultural level of DMSO to a final concentration of 2 μM/mL. The PMA solution was dispensed

in centrifuge tubes and stored at -20℃ until use. The andrographolide was dissolved with the same method as above and made a final concentration of 50 mM/mL. We diluted the andrographolide solution into 42, 28, 14 mM/mL, corresponding to high dose (42 mM/mL), medium dose (28 mM/mL) and low dose (14 mM/mL) separately. The andrographolide solution prepared well should be stored at -20℃ until use.

Cell culture

Primary HUVECs were obtained from ATCC (Rockville, MD, USA). High glucose-DMEM medium, supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), used as maintenance medium to culture HUVECs in an incubator with 5% CO_2, at 37℃. All the cells must be cultured at approximately 90% confluence before the experiment.

HUVECs cultured in 96-well plates were divided into the control group, positive group, drug group and high, medium and low dose three treatment groups. Each group set three duplicate wells and each experiment set three parallels test. The concrete operation for cells treatment was as followed: Endothelial cells were cultured in serum-free medium for 12 h before the formal experiment. After the starvation, the drug group and three treatment groups were pretreated with andrographolide solution to a final concentration of 42, 14, 28, 42 μM/mL for 12 h. Then the positive group and three treatment groups were treated with 2 μM/mL PMA for 1 h.

Cell viability

MTS assay was applied to assess the cell viability. Each well of 96-well plates was added 100 μL cell suspension and the plates were pre-cultured for 24 h (at 37℃, with 5% CO_2). Then cells were treated with increasing concentrations of AND (1-50 μM/mL) for 24 h. 20 μL MTS solution (2.5mg/mL in PBS) and 180 μL culture medium were added to each well and continuously incubated for 2.5 h. Finally, the result of each well optical density was measured at 490 nm with microplate reader.

ELISA for sEPCR

ELISA kits were used to examine concentrations of sEPCR. The testing samples were prepared from cell culture media. Firstly, per well of the 96-well plate was added with 100 μL samples. Then, the plate sealed with adhesive cover was incubated at 37℃ for 90 min, after which the contents in plate were removed. 100 μL of biotinylated anti-human EPCR antibody working solution was added per well in the plate, which later was incubated for 60 min at 37℃. The plate then was washed for three times using 0.01M Tris-buffered saline (TBS) and the liquid must be removed completely at the last time. Next, the plate adding with 100 μL of BCA working solution per well was incubated for 30 min at 37℃ and then the plate was washed for five times totally. Subsequently, the plate was added with 90 μL of TMB color-developing agent per well and incubated 20 min at 37℃ in darkness. Following incubation, 100 μL of TMB stop solution was added to terminate reactions. Finally, the optical density of samples at 450nm was measured by the microplate reader. The concentrations of the samples were

calculated from the standard curve.

Western blot for EPCR, TACE and MAPKs

The protein lysates included total proteins and membrane proteins were extracted from homogenizing HUVECs by two lysis buffers (AR0102 and AR0155) (Boster, Wuhan, PR China) separately. BCA Protein Assay Kit (Beyotime, Jiangsu, PR China) was used to detect the protein concentration. Equal amounts of proteins (15μg/well) were separated by SDS-PAGE (10%), and then transferred to polyvinylidene fluoride (PVDF) membranes. 0.5% skim milk severed as the sealant, which blocked membranes with sustained shaking for 1 h at room temperature (r.t.). After that, membranes separately incubated with primary antibodies (monoclonal antibody of EPCR 1:2000 dilution, TACE 1:1000 dilution, Phospho-MAPK family antibody 1:2000 dilution, MAPK family antibody 1:2000 dilution and anti-β-actin 1:15,000 dilution) at 4℃ overnight. Following incubation, membranes were washed with Tris-buffered saline tween-20 (TBST) for three times (5 min/time) and incubated with secondary antibody (HRP-labeled Goat anti-Mouse or Rabbit IgG, 1:5,000 dilution) at r.t. for 1 h. Subsequently, membranes were washed with TBST and developed with ECL-plus system (Bio-Rad, Hercules, CA, U.S.A.). Equivalent proteins were confirmed by expression of anti-β-actin and density analysis of western blot bands was achieved by Alpha image software (Alpha Innotech, Santa Clara, CA, U.S.A.).

Quantitative RT-PCR for TACE

Total RNA was extracted from HUVECs using TRI-Reagent (Invitrogen, Grand Island, NY) according to instructions. cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). The real-time polymerase chain reactions were conducted applying iQ SYBR Green Super Mix (Bio-Rad). The quantitative RT-PCR performed expression of TACE and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA through the CFX Connect ™ Real-Time PCR Detection System (Bio-Rad). The primer sequences were as followed: TACE, 5'-ACC TGA AGA GCT TGT TCA TCG AG-3' (forward) and 5'-CCA TGA AGT GTT CCG ATA GAT GTC-3' (reverse); GAPDH, 5'-TCG GAG TCA ACG GAT TT-3' (forward) and 5'-CCA CGA CGT ACT CAG C-3' (reverse). Gene amplification was as followed: 95℃ for 3 min, followed by 95℃ for 15 s and 60℃ for 30 min for 40 cycles. Relative gene expression levels were calculated using the expression level of GAPDH as an internal control.

TACE activity assay

TACE activity was detected by the InnoZyme TACE activity assay kit (EMD Millipore, Billerica, MA) based on instructions. Protein lysates were obtained by CytoBuster TM Protein Extraction Reagent (EMD Millipore, Billerica, MA). Briefly, designated wells were added with 100 μL samples individually. The plate sealed with adhesive cover was incubated at r.t. for 1 h with gentle shaking. After, each well was washed with 350 μL Wash buffer and then the content of each well was discarded. The plate was washed 5 times as this method and the residual liquid was removed

thoroughly at the last time. After that, each well was added with 100 μL substrates, followed by the plate was incubated at 37℃ for 4-5 h. Finally, the optical density of each well was measured at 324nm and 405nm by the microplate reader. Activity of TACE was calculated from the standard curve.

Statistical analysis

All the data were obtained from three independent experiments and shown as the mean±standard deviation (S.D.) eventually. The differences between test groups were compared by one-way ANOVAs and t-test. Least-Significant Difference (LSD) was employed as a multiple comparison test. Above statistical analysis was performed by SPSS 16.0 software (SPSS Science, Chicago, IL). Additionally, *P* values below 0.05 were regarded as statistically significant.

Result

The cytotoxic effect of andrographolide on HUVECs

The cytotoxic effect of andrographolide on HUVECs was detected with MTS assay. The cells were exposed to andrographolide (1-50 μM) for 24 h, followed by cell viability test. The data in *Fig. 1* displayed that andrographolide concentrations ranged from 1-50 μM, had no significant cytotoxicity to the HUVECs ($P>0.05$) during a 24 h treatment.

Effect of andrographolide on PMA-induced EPCR shedding in HUVECs

Based on our previous study, we used 2 μM PMA on HUVECs for 1 h to lead EPCR shedding fully. In order to explore whether andrographolide could inhibit PMA-induced EPCR shedding, the treatment groups were pretreated with andrographolide of the concentration gradient (14-42 μM) for 12 h, and then stimulated with 2 μM PMA for 1 h. The cells without any treatment were control group. The cells individually stimulated by PMA were positive group. The cells individually treated with 42 μM andrographolide were drug group. Western-blot and ELISA were separately performed to detect the amount of mEPCR on cell membranes and the sEPCR in cell culture medium. As is shown in the *Fig. 2* in contrast to positive group, andrographolide at concentrations ranging from 14-42 μM all could significantly reduce the content of sEPCR in culture media and increased the expression of mEPCR on cells ($P<0.01$). Inhibition of three andrographolide concentrations wasn't represented differences. Furthermore, there is no significant difference between control group and drug group ($P>0.05$).

Effects of andrographolide on PMA-induced expression and activity of TACE in HUVECs

TACE has been reported as a main role in mediating PMA-induced EPCR shedding (Qu et al. 2007). Since andrographolide could efficiently prevent the shedding of EPCR stimulated by PMA, we further tested whether this inhibition was owing to the effect of andrographolide on expression and activity of TACE. Setting experimental groups as above. The data in *Fig. 3A* showed that andrographolide (14-42 μM) could significantly

increase the activity of TACE comparing with positive group (P<0.01). Moreover, remarkable decrease in the expressions of TACE protein and mRNA could be observed in *Figs. 3B, 3C* and *3D* (P<0.01). However, there is no significant difference between the results of control group and drug group (P>0.05).

Effects of andrographolide on PMA-induced phosphorylation of MAPK in HUVECs

In prior reports, it is published that PMA could induce the phosphorylation of ERK1/2, p38 and JNK, which belong to the MAPK family and take part in EPCR shedding (Menschikowski et al. 2009). In order to further explore the effect of andrographolide on PMA-induced increasing of phosphorylation of MAPK, the proteins and phosphorylated proteins of ERK1/2, p38 and JNK were examined by Western blot with specific antibodies. Setting experimental groups as above. The data in *Figs. 4A* and *4B* clearly showed that, in comparison with positive group, andrographolide (14-42 μM) treatment groups could significantly reduce the phosphorylation of p38 and JNK induced by PMA (P<0.01). Regretfully, the phosphorylation of ERK1/2 in andrographolide treatment groups didn't perform lessen like that (P>0.05). In addition, there is no significant difference between the results of control group and drug group (P>0.05).

Discussion

EPCR participates in PC pathway, which relates to anticoagulant, anti-inflammation

and vascular barrier protection, and has been proved to prevent thrombosis in vivo directly (Centelles et al. 2010). EPCR can be shed from the cell surface into plasma as a soluble form, the improvement of sEPCR level was discovered in some infectious diseases and autoimmune diseases (Bai et al. 2020; Zhu et al. 2015; Chiappetta et al. 2016). In recent years, much attention has been appealed to the roles of EPCR in differential pathophysiology like the cancer and malaria, because of the ligands diversity (Pendurthi and Rao. 2018). Consequently, protecting EPCR has been a new idea of treating certain diseases. Prior studies illustrated PMA could accelerate the shedding of EPCR (Xu et al. 2000). In this experiment, we used PMA as inducer, and observed that andrographolide could significantly inhibit EPCR shedding, but have no influence using individually. The results showed andrographolide could protect EPCR in vitro and prompted it maybe have the potential therapeutic value in diseases with EPCR shedding. Interestingly, in recent researches the suppressive effects of several natural medicine on EPCR shedding were supposed to correspond with the anti-inflammation effects on human endothelial cells (Ku et al. 2017; Kang et al. 2016). It suggested andrographolide probably has similar function.

TACE mediates PMA-induced EPCR shedding has been confirmed previously (Qu et al. 2007). In our study, andrographolide decreased the expression levels of TACE mRNA and protein as well as the inhibition of TACE activity in endothelial cells, which evidence andrographolide serves the function of protecting EPCR on cells surface maybe through TACE depression.

MAPKs are the serine/threonine protein kinases widely distributed in eukaryotic organism. Among the more classic ones include JNK and p38, which mainly regulate inflammatory responses, and the ERK majoring in the cell proliferation and differentiation (Kim and Choi. 2015). Previous investigations of EPCR shedding have made it clear that the MAPK pathway takes part in this process through adjusting TACE (Lee et al. 2016; Menschikowski et al. 2009). In our study, we also repeated this result, the expression of MAPK phosphoproteins in model cells had been increased. In addition, we used andrographolide to process cellls and found that it shown dramatic reductions in phosphorylation levels of JNK and p38, but didn't affect the ERK. Yet, compared to the other literature focusing on the inhibition of andrographolide on MAPK pathway, which displayed all these three kinases phosphorylation were restrained (Yuan et al. 2018; Li et al. 2017; Jing et al. 2019), the differences existing between the results may be due to the various materials and methods applied in experiments.

According to Traditional Chinese Medicine, *Andrographis paniculata* (*A. paniculata*) is known for antipyretic and antidotal drug, which cold in property, bitter in flavor and belongs to the Heart, Lung, Large intestine and Urinary bladder Channel. China Pharmacopoeia stipulates above-ground part of *A. paniculata* as the medicine position (Zhang et al. 2018). Andrographolide mainly exists in the medicine part of *A. paniculata* and has been applied to some inflammatory diseases for its anti-inflammation activity. Additionally, the pharmaceutical functions of anti-cancer, antivirus and antihypertensive et al in andrographolide have drawn extensive attention (Dai et al. 2019). *A. paniculata* with high content of andrographolide is widely planted

in China. For the multiple advantages of andrographolide, like its rich resources, low toxicity and various activities, further study for andrographolide is helpful to explain its pharmacological functions and explore its clinical applied potentials, which is necessary. In our study, we confirmed andrographolide was nontoxic to cells and played a key role in protecting EPCR of HUVECs. To some degree, this finding was consistent with the other studies of andrographolide.

However, it's worth mentioning there has the bigger limitation in our experiment. We used HUVECs as research objects and the results only could prove the well EPCR protection of andrographolide in vitro. In consideration of the complexity for organisms, it's unknown whether andrographolide still can play the same role in vivo. For this reason, our study just provides certain perspective of andrographolide for its application, and its feasibility needs further exploration.

Conclusion

Collectively, the results of our study show that andrographolide efficiently inhibit the shedding of EPCR induced by PMA in HUVECs and down-regulated the expression and activity of TACE. Furthermore, the suppressions of JNK and p38 phosphorylation in MAPK signal also were detected. According to the reports of the relationships between MAPK, TACE and EPCR, we suppose that perhaps the JNK and p38 are molecular targets of andrographolide and are responsible for its anti-EPCR shedding effect (*Fig. 5*). Considering the importance of EPCR in many diseases, our findings provide new insights into the potential clinical therapeutic action of andrographolide in

some pathologic reactions, especially the vascular inflammatory diseases and thrombotic diseases.

Declarations

Ethical Approval

The human umbilical vein endothelial cells we used in this study performed all in vitro cell experiments. So, Ethics approval was not required for the present study.

Consent to Participate

Not applicable.

Consent to Publish

All authors approved the manuscript to be published.

Authors Contributions

HCL, LXX and YLZ contributed to the study conception and design. Material preparation was performed by LXX and YLZ. Data collection and analysis were performed by HCL and WZY. The first draft of the manuscript was written by WZY. All authors read and approved the manuscript and all data were generated in-house and that no paper mill was used.

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Availability of data and materials

Upon request.

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Fig. 1 The cytotoxicity effect of andrographolide on HUVECs

The cells were pretreated with andrographolide (1-50 μM) for 24 h, and then MTS assay was applied to measure the cell viability. For comparison, cells were also incubated in a media without andrographolide.

Fig. 2 The effect of andrographolide on PMA-induced EPCR shedding in HUVECs

The cells were pretreated with andrographolide (14-42 μM) for 12 h and then incubated with 2 μM PMA for 1 h. The expressions of sEPCR and mEPCR were detected by ELISA (A) and WB (B). C performed the corresponding densitometric-measurement of western blot bands. $^{*}P<0.01$ versus the positive group, $^{\#}P<0.01$ versus the Control group.

Fig. 3 Effects of andrographolide on PMA-induced expression and activity of TACE in HUVECs

The cells were pretreated with andrographolide (14-42 μM) for 12 h and then incubated with 2 μM PMA for 1 h. A was the TACE activity assay, and the expressions of TACE mRNA and protein were detected by qRT-PCR (B) and WB (C). Densitometric analysis was used to implement the protein quantification (D). $^{*}P<0.01$ versus the positive group, $^{\#}P<0.01$ versus the Control group.

Fig. 4 Effects of andrographolide on PMA-induced phosphorylation of MAPK in HUVECs

The cells were pretreated with andrographolide (14-42 μM) for 12 h and then incubated with 2 μM PMA for 1 h and were detected by WB (A) . Densitometric measurement was served to Protein quantification (B). * $P<0.01$ versus the positive group, $^{\#}$ $P<0.01$ versus the Control group.

Fig. 5 The model of 'Andrographolide inhibits PMA-induced EPCR shedding through JNK and p38 MAPK'

Figures

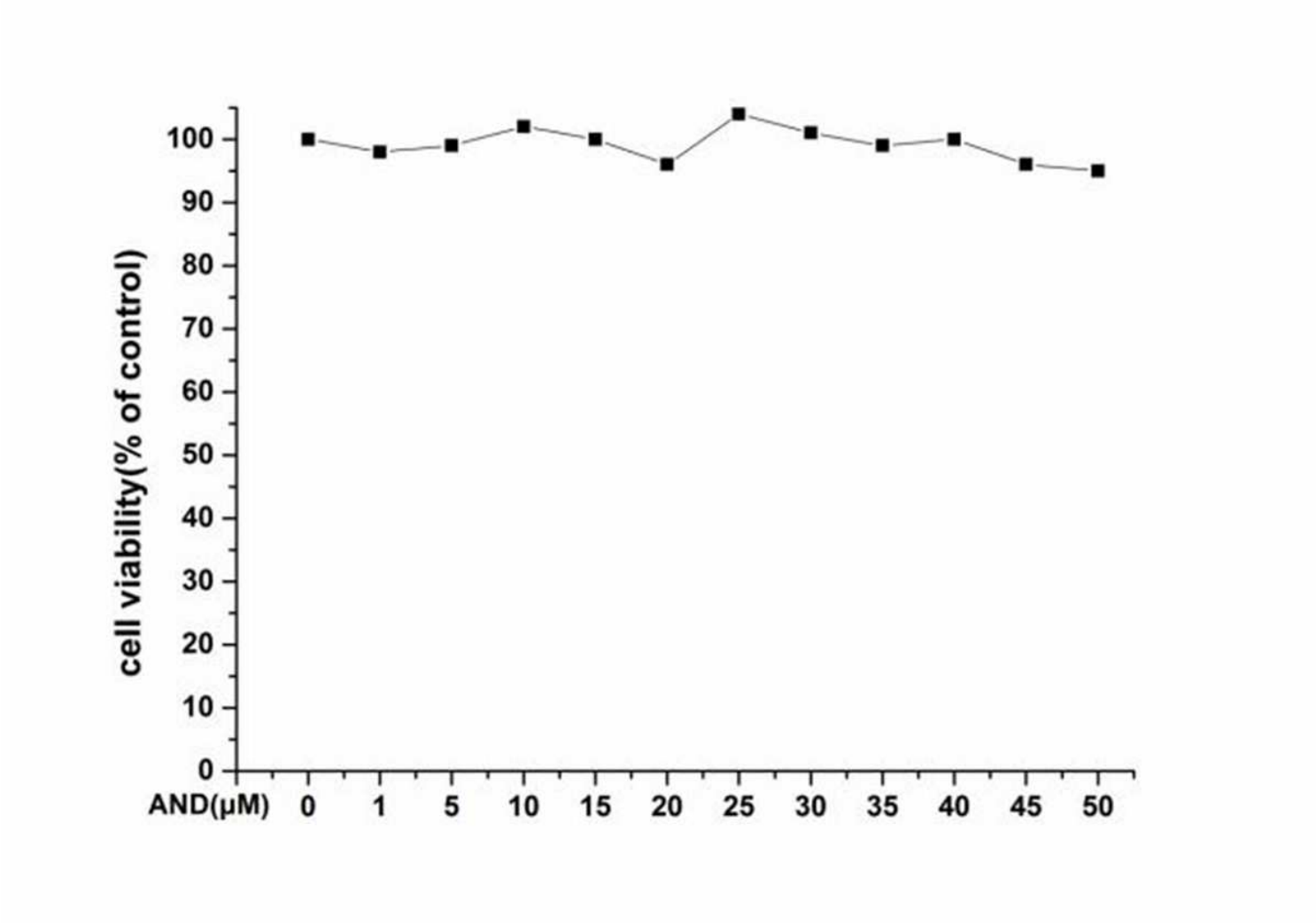

Figure 1

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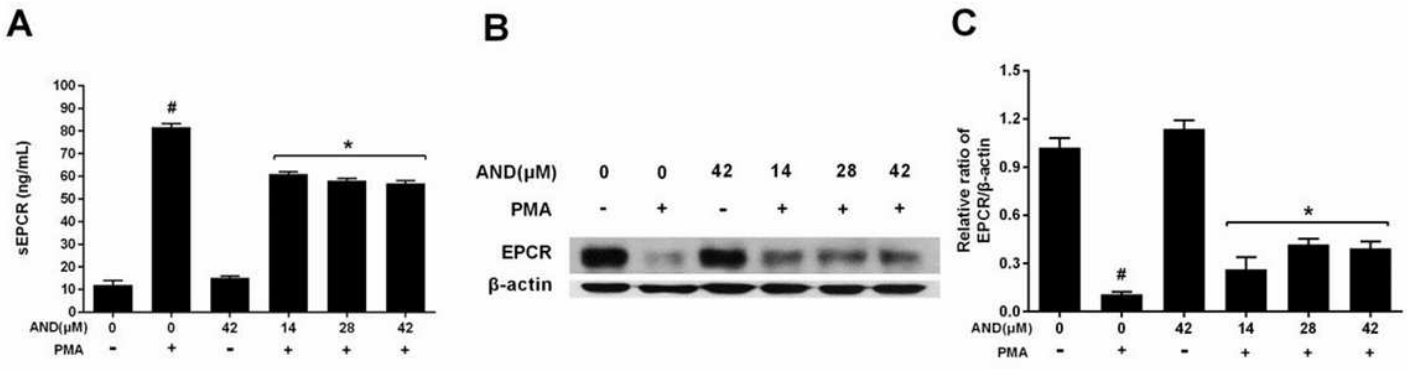

Figure 2

The effect of andrographolide on PMA-induced EPCR shedding in HUVECs The cells were pretreated with andrographolide (14-42 μM) for 12 h and then incubated with 2 μM PMA for 1 h. The expressions of sEPCR and mEPCR were detected by ELISA (A) and WB (B). C performed the corresponding densitometric-measurement of western blot bands. *P<0.01 versus the positive group, # P<0.01 versus the Control group.

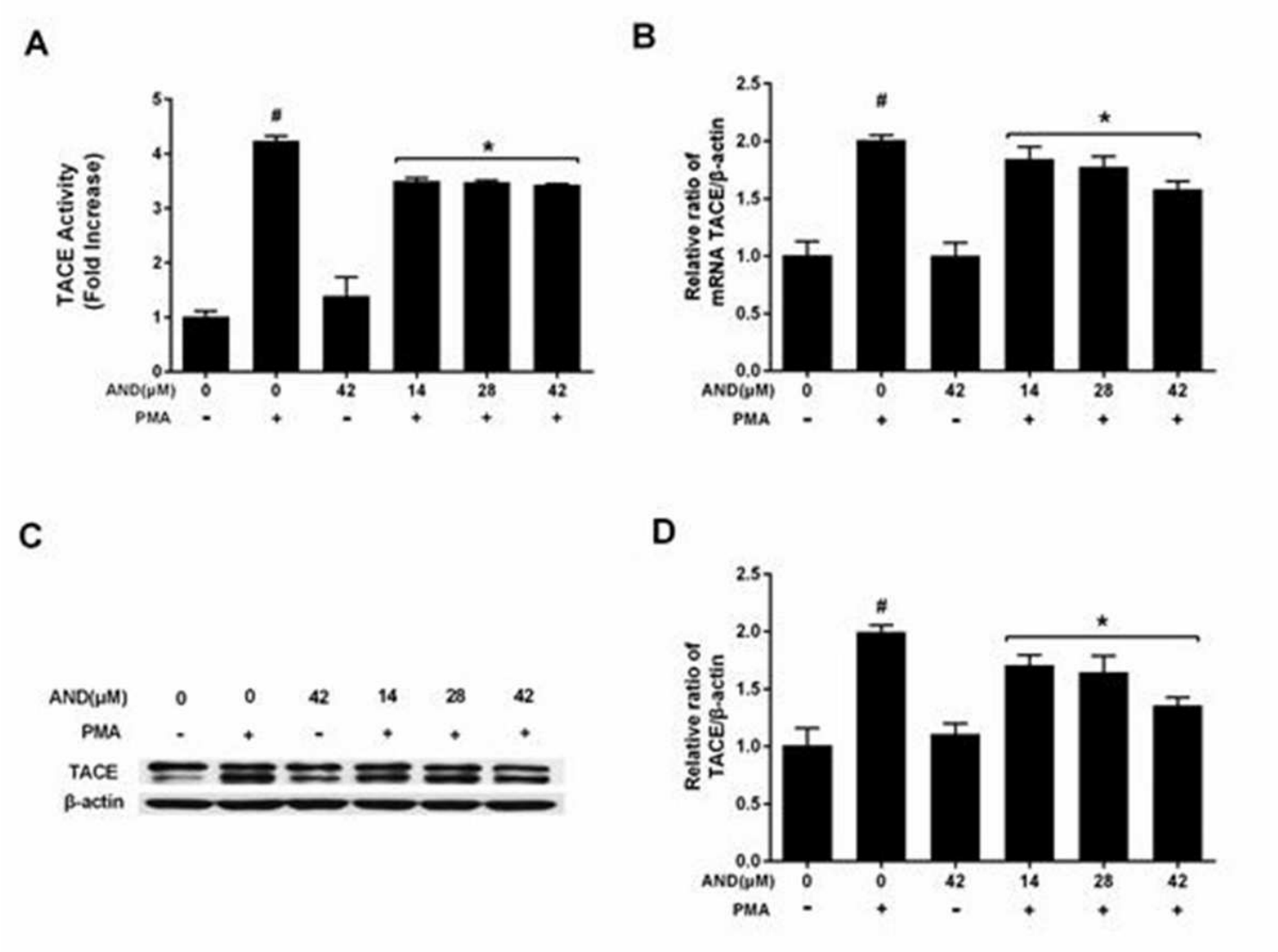

Figure 3

Effects of andrographolide on PMA-induced expression and activity of TACE in HUVECs The cells were pretreated with andrographolide (14-42 μM) for 12 h and then incubated with 2 μM PMA for 1 h. A was the TACE activity assay, and the expressions of TACE mRNA and protein were detected by qRT-PCR (B) and WB (C). Densitometric analysis was used to implement the protein quantification (D). * P<0.01 versus the positive group, # P<0.01 versus the Control group.

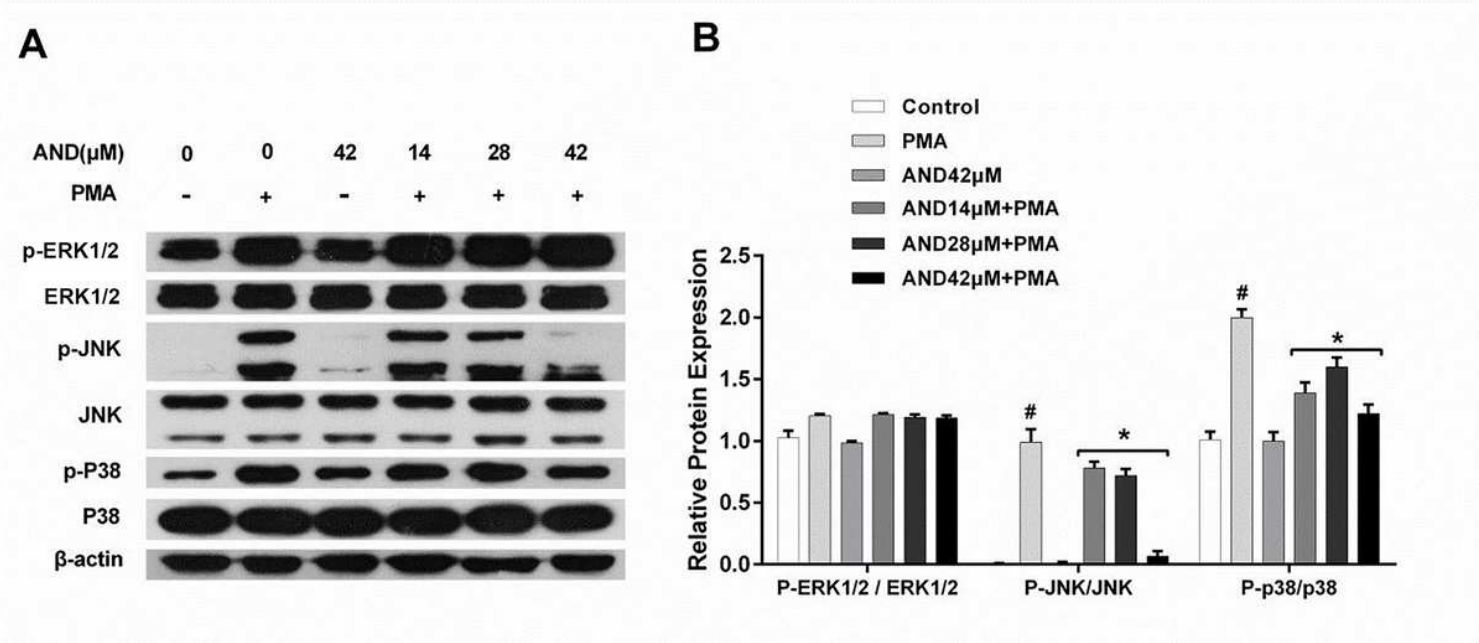

Figure 4

Effects of andrographolide on PMA-induced phosphorylation of MAPK in HUVECs The cells were pretreated with andrographolide (14-42 μM) for 12 h and then incubated with 2 μM PMA for 1 h and were detected by WB (A) . Densitometric measurement was served to Protein quantification (B). * P<0.01 versus the positive group, # P<0.01 versus the Control group.

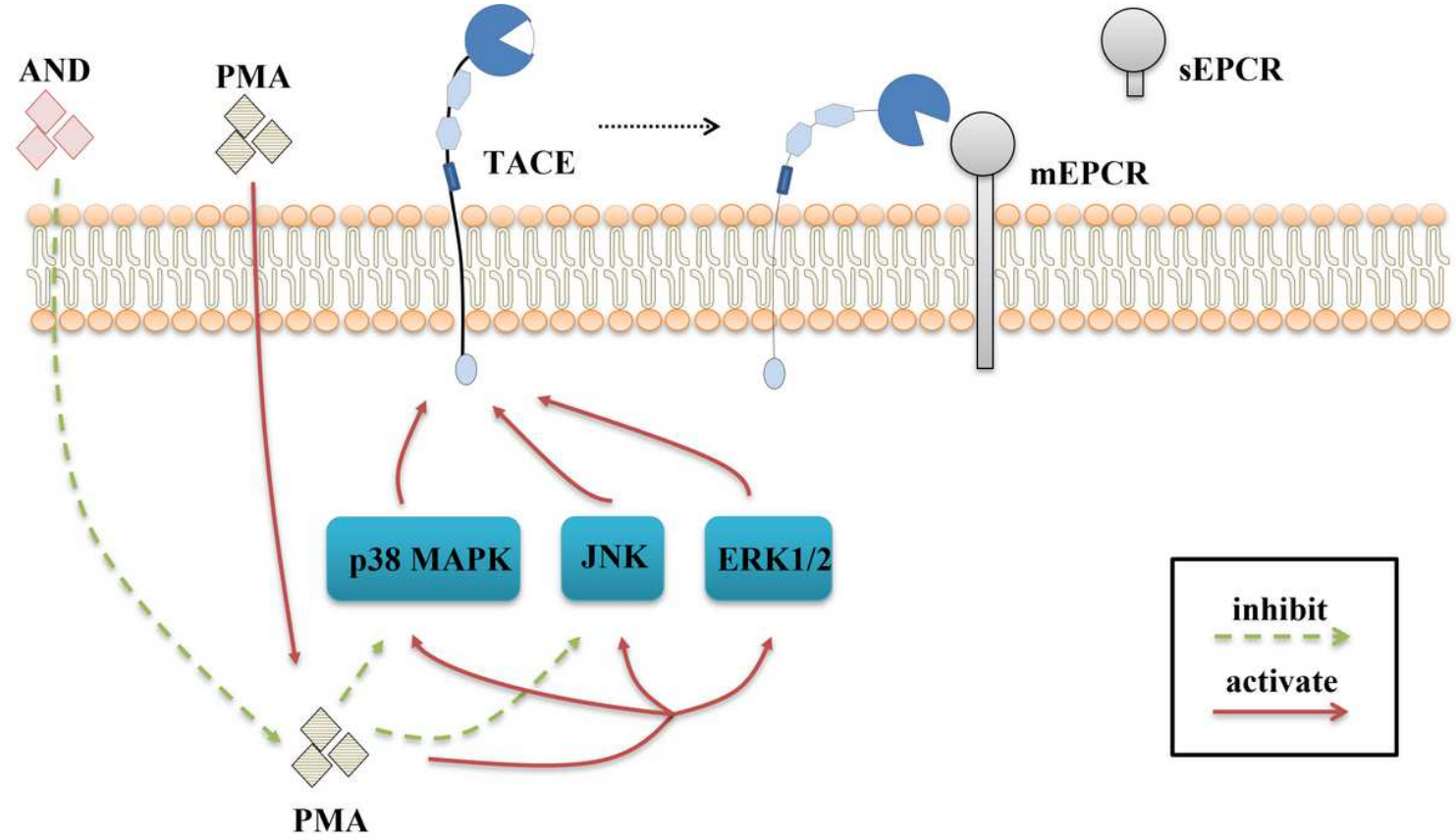

Figure 5

The model of ‘Andrographolide inhibits PMA-induced EPCR shedding through JNK and p38 MAPK'

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- ELISAsEPCR.xls
- MTSassay.xlsx