Cellular Signalling 26 (2014) 220–232

Contents lists available at ScienceDirect

Cellular Signalling

journal homepage: www.elsevier.com/locate/cellsig

# Tissue kallikrein mediates neurite outgrowth through epidermal growth factor receptor and flotillin-2 pathway in vitro

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ARTICLE INFO

*Article history:*
Received 29 July 2013
Received in revised form 8 October 2013
Accepted 31 October 2013
Available online 6 November 2013

*Keywords:*
Tissue kallikrein
Neurite outgrowth
Epidermal growth factor receptor
Flotillin-2
Extracellular signal-regulated kinase

ABSTRACT

Tissue kallikrein (TK) was previously shown to take most of its biological effects through bradykinin receptors. In this study, we assumed that TK mediated neurite outgrowth was independent of bradykinin receptors. To test the hypothesis, we investigated TK-induced neurite outgrowth and its signaling mechanisms in cultured primary neurons and human SH-SY5Y cells. We found that TK stimulation could increase the number of processes and mean process length of primary neurons, which were blocked by epidermal growth factor receptor (EGFR) inhibitor or down-regulation, small interfering RNA for flotillin-2 and extracellular signal-regulated kinase (ERK) 1/2 inhibitor. Moreover, TK-induced neurite outgrowth was associated with EGFR and ERK1/2 activation, which were inhibited by EGFR antagonist or RNA interference and flotillin-2 knockdown. Interestingly, inhibition of bradykinin receptors had no significant effects on EGFR and ERK1/2 phosphorylation. In the present research, our data also suggested that EGFR and flotillin-2 formed constitutive complex that translocated to around the nuclei in the TK stimulation. In sum, our findings provided evidence that TK could promote neurite outgrowth via EGFR, flotillin-2 and ERK1/2 signaling pathway in vitro.



## 1. Introduction

Tissue kallikrein (TK), an important component of the kallikrein–kinin system (KKS), belongs to a subgroup of serine proteinases and processes low molecular weight (LMW) kininogen to release kinin peptide [1,2]. Most of the cellular effects of TK are thought to be mediated by bradykinin (BK), which acts via G protein-coupled B1 and B2 bradykinin receptors (B1R and B2R). However, recent findings revealed that TK could directly activate B2R independent of kininogen and kinin release in cultured CHO cells [3,4]. Moreover, TK was found to trigger the proteinase-activated receptor 1 ($PAR_1$) signaling pathway and epidermal growth factor receptor (EGFR) transactivation in HaCaT keratinocyte cells [5]. These combined information indicated that actions of TK might contribute to other signaling events in addition to BK pathway.

EGFR is a transmembrane receptor comprising a family of classical receptor tyrosine kinases that trigger a rich network of signaling pathways to control cell survival and growth [6,7]. There are at least 11 endogenous EGFR ligands, each of which is synthesized as precursors and cleaved to generate the mature one. Ligand binding results in multiple phosphorylation events of EGFR, which in many cases are mediated by tyrosine kinases. Simultaneous activation of downstream cascades, such as the mitogen-activated protein kinase (MAPK) pathway, translates in the nucleus into distinct transcriptional programs. Furthermore, previous research showed that lipid rafts were involved in the signaling and/or trafficking of EGFR [8,9].

Reggie-1/flotillin-2 (Flot2) and reggie-2/flotillin-1 (Flot1) are two highly conserved lipid-raft-associated proteins, which were originally discovered as proteins up-regulated in retinal ganglion cells during axon regeneration [10,11]. Flotillins share an SPFH (Stomatin/Prohibitin/Flotillin/HflK/C) domain at their N-terminus containing residues for myristoylation and palmitoylation and thus membrane anchorage [12,13]. The C-terminus of reggies harbors a unique flotillin domain that is predicted to adopt an alpha-helical coiled-coil structure [10,11]. Even though the function of flotillins has remained elusive and controversial, their widespread expression and conservation imply that these proteins regulate basic cellular processes. Evidence is accumulating that flotillins are involved in the signaling processes of several membrane receptors, phagocytosis, non-clathrin endocytosis, and organization of the actin cytoskeleton [14–20]. They have also been suggested to function as key proteins for neurite outgrowth [21,22]. In

*Abbreviations:* TK, tissue kallikrein; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; KKS, kallikrein–kinin system; BK, bradykinin; B1R, B1 bradykinin receptor; B2R, B2 bradykinin receptor; PAR, proteinase-activated receptor; Flot, flotillin; DIV, days in vitro.

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http://dx.doi.org/10.1016/j.cellsig.2013.10.010

addition, recent studies have demonstrated that flotillins were modified by EGFR signaling, and that Flot2 became phosphorylated at several tyrosine residues and translocated from plasma membrane into endosomes in the stimulation of EGFR [15].

Although such molecular effects of flotillins have been reported, the role of Flot2 in TK-induced neurite outgrowth has received relatively little attention. Here, we describe a novel neurite growth mechanism whereby TK activates the extracellular signal-regulated kinase (ERK) 1/2 pathway through EGFR and Flot2 independent of BK receptors.

## 2. Materials and methods

### 2.1. Materials

TK was from Techpool Bio-Pharma Co. (Guangzhou, China). EGFR, phospho-EGFR (Tyr1068), ERK1/2 (Thr202/Tyr204), phospho-ERK1/2 (Thr202/Tyr204), Flot2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibodies were from Cell Signaling Technology (Beverly, MA, USA). Affinity-purified rabbit anti-microtubule-associated protein 2 (MAP2) antibody was from Sigma-Aldrich (St. Louis, MO, USA). B1R polyclonal antibody and B2R monoclonal antibody were from Abcam (Cambridge, UK). Secondary antibodies coupled to HRP were from Cell Signaling Technology (Beverly, MA, USA). Alexa Fluor 488- and 555-conjugated secondary antibodies were from Invitrogen (Grand Island, NY, USA). Lys-(des-Arg9, Leu8)-BK (DALBK), HOE140, aprotinin, AG1478, and PD98059 were from Sigma-Aldrich (St. Louis, MO, USA). The Lipofectamine 2000 transfection reagent and protein G Dynabeads were from Invitrogen (Grand Island, NY, USA).

### 2.2. Cell cultures and transfections

All of the procedures were performed according to the NIH Guide for the Care and Use of Laboratory Animals as well as Fudan University experimental standards. Primary neurons were prepared from embryonic BALB/c mice. Briefly, cortex was isolated, digested, and homogenized. Cells were plated on poly-L-lysine-coated coverslips in Neurobasal-A medium containing B-27 supplement, 0.5 mM L-glutamine and 1% penicillin–streptomycin (Invitrogen). Cultured primary neurons from 3 days in vitro (DIV) were transfected as described previously [22]. Mouse Flot2 and EGFR RNA interference target sequences for primary neurons were shown in Table 1. The negative control (NC) siRNA with non-targeting sequence for mouse was from Invitrogen. Experiments were performed at 48 h after transfection.

Human SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) at 37 °C and 5% $CO_2$. RNA interference target sequences against human Flot2, B1R, B2R and EGFR for SH-SY5Y cells were shown in Table 1. The commercial siRNA from Invitrogen with non-targeting sequence was used as a non-specific control. SH-SY5Y cells were transiently transfected with Lipofectamine 2000 according to the manufacturer's instructions, and experiments were carried out at 48 h after transfection.

### 2.3. Cell experiment treatment

For drug treatment, primary neurons were exposed to TK at 1 μM for 48 h, and SH-SY5Y cells were serum-starved overnight before various concentration of TK (0.25 to 1 μM) were given for 5, 15 or 30 min. To study the involvement of BK receptors on TK effects, B1R antagonist DALBK (1 μM) or B2R antagonist HOE140 (10 μM) was added 1 h prior to TK treatment. TK was inactivated by pre-incubation with five-fold molar excess of aprotinin for 1 h at 37 °C [23]. In some experiments cells were treated with EGFR inhibitor AG1478 (300 nM) or ERK kinase inhibitor PD98059 (10 μM) for 1 h previous to TK stimulation.

### 2.4. Co-immunoprecipitation

Starved SH-SY5Y cells were either stimulated with 1 μM TK for 5 min or left untreated and then lysed in NP-40 lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40 and protease inhibitor mixture) supplemented with 1 mM PMSF. Antibody against Flot2 (diluted 1:100) or EGFR (diluted 1:100) was coupled on protein G Dynabeads. The lysates and beads were rolled overnight at 4 °C, and then the beads were washed 3 times in washing buffer. The beads and lysates were supplemented with SDS sample buffer and heated at 94 °C for 5 min.

### 2.5. Western blotting

The cells were lysed in RIPA lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS containing phosphatase and protease inhibitor mixture) supplemented with 1 mM PMSF on ice for 30 min. Protein concentration was determined using the BCA kit. Equal amounts of protein were analyzed by 8% or 10% SDS-PAGE and were transferred to PVDF membrane (Millipore). Immunoblotting was performed with the appropriate primary antibody followed by HRP-conjugated secondary antibody. Antibody against p-EGFR (diluted 1:1000), EGFR (diluted 1:1000), p-ERK1/2 (diluted 1:2000), ERK1/2 (diluted 1:1000), Flot2 (diluted 1:1000), B1R (diluted 1:500), B2R (diluted 1:1000) or GAPDH (diluted 1:10000) was used in the corresponding experiments. Blotted proteins were visualized using an enhanced chemiluminescence assay.

Western blot bands of phosphorylated proteins were quantified by scanning densitometry using Scion Image software and normalized against the total amount of the respective protein unless stated, otherwise GAPDH was used as an equal loading control.

**Table 1**
RNA interference target sequences.

| siRNA | Sense | Antisense | Vendor |
|---|---|---|---|
| Mouse Flot2 | GUUCAUGGCAGACACCAAGTT | CUUGGUGUCUGCCAUGAACTT | Invitrogen |
| | GGUGAAGAUCAUGACGGAGTT | CUCCGUCAUGAUCUUCACCTT | Invitrogen |
| | GGUUUAUAGGCCUUCUUCCTT | GGAAGAAGGCCUAUAAACCTT | Invitrogen |
| Mouse EGFR | GGGAACUGCCCAUGCGGAATT | UUCCGCAUGGGCAGUUCCCTT | Invitrogen |
| | CCAUCAAGGAGUUAAGAGATT | UCUCUUAACUCCUUGAUGGTT | Invitrogen |
| | GAAUAUUAAGCAGCAUUUATT | UAAAUGCUGCUUAAUAUUCTT | Invitrogen |
| Human Flot2 | GUUCAUGGCAGACACCAAGTT | CUUGGUGUCUGCCAUGAACTT | Invitrogen |
| | GGUGAAGAUCAUGACGGAGTT | CUCCGUCAUGAUCUUCACCTT | Invitrogen |
| Human B1R | CUGCCAACAUUUAUCAUCUTT | AGAUGAUAAAUGUUGGCAGTT | Santa Cruz |
| | CAAGGAUUGUGGAGUUAAATT | UUUAACUCCACAAUCCUUGTT | Santa Cruz |
| | CUGCGAUCGUCUUCUUCAATT | UUGAAGAAGACGAUCGCAGTT | Santa Cruz |
| Human B2R | UGCCAUUAUCUCCAUGAACTT | GUUCAUGGAGAUAAUGGCATT | GenePharma |
| | GGGAAGUGUUCACCAACAUTT | AUGUUGGUGAACACUUCCCTT | GenePharma |
| Human EGFR | GGCUCUGGAGGAAAAGAAATT | UUUCUUUUCCUCCAGAGCCTT | GenePharma |