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RESEARCH PAPER

Endothelin-converting enzyme 1 promotes re-sensitization of neurokinin 1 receptor-dependent neurogenic inflammation

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Background and purpose: The metalloendopeptidase endothelin-converting enzyme 1 (ECE-1) is prominently expressed in the endothelium where it converts big endothelin to endothelin-1, a vasoconstrictor peptide. Although ECE-1 is found in endosomes in endothelial cells, the role of endosomal ECE-1 is unclear. ECE-1 degrades the pro-inflammatory neuropeptide substance P (SP) in endosomes to promote recycling and re-sensitization of its neurokinin 1 (NK_1) receptor. We investigated whether ECE-1 regulates NK_1 receptor re-sensitization and the pro-inflammatory effects of SP in the endothelium.
Experimental approach: We examined ECE-1 expression, SP trafficking and NK_1 receptor re-sensitization in human microvascular endothelial cells (HMEC-1), and investigated re-sensitization of SP-induced plasma extravasation in rats.
Key results: HMEC-1 expressed all four ECE-1 isoforms (a-d), and fluorescent SP trafficked to early endosomes containing ECE-1b/d. The ECE-1 inhibitor SM-19712 prevented re-sensitization of SP-induced Ca^{2+} signals in HMEC-1 cells. Immunoreactive ECE-1 and NK_1 receptors co-localized in microvascular endothelial cells in the rat. SP-induced extravasation of Evans blue in the urinary bladder, skin and ears of the rat desensitized when the interval between two SP injections was 10 min, and re-sensitized after 480 min. SM-19712 inhibited this re-sensitization.
Conclusions and implications: By degrading endocytosed SP, ECE-1 promotes the recycling and re-sensitization of NK_1 receptors in endothelial cells, and thereby induces re-sensitization of the pro-inflammatory effects of SP. Thus, ECE-1 inhibitors may ameliorate the pro-inflammatory actions of SP.
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Keywords: endothelin-converting enzyme 1; substance P; neurokinin 1 receptor; neurogenic inflammation

Abbreviations: CGRP, calcitonin gene-related peptide; ECE-1, endothelin-converting enzyme 1; EEA1, early endosomal antigen 1; ET, endothelin; HMEC-1, human dermal microvascular endothelial cells; NK_1 receptor, neurokinin 1 receptor; NEP, neprilysin, neutral endopeptidase EC 3.4.24.11; SP, substance P

Introduction

Endothelin-converting enzyme 1 (ECE-1) is a zinc metalloendopeptidase that exists as four isoforms (a-d), which share a common catalytic domain. They differ only in their amino-terminus, which determines their subcellular distribution: ECE-1a and ECE-1c are predominantly localized at the plasma membrane, whereas ECE-1b and ECE-1d are mostly found in endosomes (Schweizer *et al.*, 1997; Azarani *et al.*, 1998; Muller *et al.*, 2003). The ECE-1 isoforms are generated from a single gene through different promoters that regulate the expression of the unique amino-terminus (Valdenaire *et al.*, 1995; 1999; Meidan *et al.*, 2005).

The role of ECE-1 in the cardiovascular system has been thoroughly investigated (see Turner and Murphy, 1996). Within endothelial cells, ECE-1 converts inactive big endothelin (big ET) to the vasoactive peptide ET-1, which is secreted from cells onto underlying vascular smooth muscle (Xu *et al.*, 1994; Russell *et al.*, 1998; Russell and Davenport, 1999). Although newer biological roles for ECE-1 are emerging, its function in endosomes is not fully understood. However, ECE-1 efficiently hydrolyzes neuropeptides such as bradykinin, calcitonin gene-related peptide (CGRP), somatostatin, substance P (SP) and neurotensin at a pH similar to that found in endosomes (Hoang and Turner, 1997; Johnson *et al.*, 1999; Fahnoe *et al.*, 2000; Padilla *et al.*, 2007; Roosterman *et al.*, 2007; 2008). Thus, ECE-1 may degrade endocytosed

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neuropeptides to regulate trafficking and signalling of internalized receptors.

ECE-1 shares structural identity and sequence homology with neprilysin (neutral endopeptidase-24.11, NEP, EC 3.4.24.11) (Turner *et al.*, 2001). By degrading neuropeptides at the cell surface, NEP terminates the pro-inflammatory effect of SP (Matsas *et al.*, 1984; Lu *et al.*, 1997). SP belongs to the tachykinin family of neuropeptides and co-localizes with CGRP in the C fibre subpopulation of primary sensory nerves, which are also involved in neurogenic inflammation and pain transmission. Numerous noxious and inflammatory stimuli activate sensory nerve endings to release SP and CGRP. SP interacts with neurokinin 1, 2 and 3 receptors (NK receptors; nomenclature follows Alexander *et al.*, 2008), albeit with graded affinity. Activation of the high affinity NK_1 receptor on endothelial cells of post-capillary venules and collecting venules rapidly induces the formation of intercellular gaps in the wall of the vessels, through which plasma proteins, fluid and neutrophils can extravasate (McDonald *et al.*, 1996). CGRP induces vasodilatation and hyperaemia (Brain *et al.*, 1985). SP-induced plasma extravasation and CGRP-induced hyperaemia are prominent features of neurogenic inflammation, which contributes to the pathogenesis of inflammatory diseases and pain syndromes, including asthma, migraine, inflammatory bowel disease, arthritis and psoriasis. Mechanisms that terminate neurogenic inflammation include neprilysin degradation of SP to prevent NK_1 receptor activation (Matsas *et al.*, 1984; Lu *et al.*, 1997), and NK_1 receptor desensitization and endocytosis (Bowden *et al.*, 1994). Recycling of the NK_1 receptor to the plasma membrane mediates re-sensitization of receptors and of the pro-inflammatory effects of SP (Roosterman *et al.*, 2004).

Although the molecular mechanisms of NK_1 receptor desensitization and endocytosis have been thoroughly examined (McConalogue *et al.*, 1998), little is known about the mechanisms of NK_1 receptor recycling and re-sensitization. We recently reported that ECE-1 degrades SP and CGRP in acidified endosomes to metabolites that are unable to re-bind to and activate the receptor (Padilla *et al.*, 2007; Roosterman *et al.*, 2007). This degradation disrupts the peptide-receptor-β-arrestin complex, allowing β-arrestins to return to the cytosol and initiating receptor recycling and re-sensitization. This is a new mechanism by which endosomal ECE-1 regulates post-endocytic sorting of the receptors, thereby promoting receptor recycling and re-sensitization. In the current study, we examined the role of ECE-1 in regulating NK_1 receptor re-sensitization in endothelial cells and in controlling re-sensitization of the pro-inflammatory effects of SP. Our aims were to (i) determine whether ECE-1 and NK_1 receptors are co-expressed in microvascular endothelial cells that are sites of plasma extravasation; (ii) examine whether SP is endocytosed to ECE-1-containing endosomes in these cells; (iii) investigate whether ECE-1 controls re-sensitization of SP-induced Ca^{2+} mobilization in microvascular endothelial cells; and (iv) determine if ECE-1 controls re-sensitization of the pro-inflammatory effects of SP. Our results show that ECE-1 promotes re-sensitization of SP-induced Ca^{2+} signalling and plasma extravasation in the endothelium of multiple tissues.

Methods

Animals

The Institutional Animal Care and Use Committee of the University of California, San Francisco, approved all animal procedures and experimental protocols. Male Sprague-Dawley rats (200–250 g) were from Charles River Laboratories (Wilmington, MA). Rats were kept in an air and temperature controlled environment with standard laboratory food, water freely available, and a 12:12 h light–dark cycle.

Cell lines and constructs

SV40 large T Ag-transformed human dermal microvascular endothelial cells (HMEC-1) were a gift from Dr John Ansel (University of Arkansas) and were grown in MCDB131 supplemented with 10% HIFBS, l-glutamine (2 mmol·L^{-1}), hydrocortisone acetate (1 μmol·L^{-1}), N6,2′-O-dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt (0.5 μmol·L^{-1}) and human epidermal growth factor (10 ng·mL^{-1}). Cells were grown in 95% air, 5% CO_2 at 37°C. cDNA expressing rat NK_1 receptors with a N-terminal HA epitope was from M. von Zastrow (University of California, San Francisco) and subcloned into pcDNA5/FRT (Invitrogen). HMEC-1 were transiently transfected with rat NK_1 receptors using an Amaxa Nucleofector System (Gaithersburg, MD) according to the manufacturer's guidelines, and studied after 48 h. HEK cells expressing ECE-1c-GFP or control vector have been described (Padilla *et al.*, 2007).

RT-PCR

Total RNA from HMEC-1 cells was isolated using Trizol (Invitrogen, Carlsbad, CA). RNA (2 μg) was reverse transcribed using standard protocols with random hexamers and TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA). Subsequent PCR reactions used primers specific for ECE-1 isoforms (forward 1a 5′-ggctgaatctgtgggaaccaga-3′; forward 1b 5′-cggtgtccgccctgctgt-3′; forward 1c 5′-gcggagcacgcgagctat-3′; forward 1d 5′-gagggagtccgtgctgcat-3′ and reverse 5′-gaggaggtg cttgatgattgcttg-3′). Control reactions omitted reverse transcriptase. The PCR products were separated by electrophoresis (1.5% agarose gel) and stained using ethidium bromide. These primers have been used previously to detect ECE-1 mRNA (Padilla *et al.*, 2007; Roosterman *et al.*, 2007).

Measurement of $[Ca^{2+}]_i$

HMEC-1-NK_1 receptor cells grown on 96-well plates were incubated with 2.5 μmol·L^{-1} Fura-2AM (Invitrogen) in HBSS (0.1% BSA; 20 mmol·L^{-1} HEPES, pH 7.4) for 20 min at 37°C and washed. Fluorescence was measured at 340 and 380 nm excitation and 510 nm emission in a Flex Station III Microplate Reader (Molecular Devices, Sunnyvale, CA). The ratio of the fluorescence at the two excitation wavelengths, which is proportional to $[Ca^{2+}]_i$, was calculated, and results are expressed as increase above basal values. To assess the role of ECE-1 on re-sensitization, HMEC-1-NK_1 receptor cells were pre-incubated with vehicle (control) or SM-19712

(10 μmol·L^{-1}, 30 min). Cells were then incubated with SP (10 nmol·L^{-1}, 10 min, 37°C), which desensitizes the NK_1 receptors (Roosterman *et al*., 2007), or vehicle (control). Cells were washed and recovered in SP-free medium for 30 min to allow re-sensitization to proceed. The change in $[Ca^{2+}]_i$ in response to a second SP challenge (10 nmol·L^{-1}) was then measured. Results are expressed as % of controls (100%).

Immunofluorescence in transfected cells

HMEC-1-NK_1 receptor cells plated 0.1% gelatine-coated glass coverslips were incubated with Alexa 546 SMSP-SMSP (100 nmol·L^{-1}, 2 h, 4°C), washed and incubated for 30 min at 37°C. Cells were fixed in 4% paraformaldehyde in 100 mmol·L^{-1} PBS, pH 7.4 (20 min, 4°C) and washed for 15 min with PBS containing 0.1% saponin and 1% normal goat serum. Cells were incubated with primary antibodies to ECE-1 (#52-6497, rabbit antibody to human ECE-1, 1:1000) and to the early endosomal antigen 1 (EEA1; 1:250, overnight, 4°C). We have previously reported that this ECE-1 antibody recognizes the ECE-1b and ECE-1d isoforms (Padilla *et al*., 2007; Roosterman *et al*., 2007). Cells were washed again and incubated with secondary antibodies conjugated to fluorescein isothiocyanate or Cy5 (1:500, 2 h room temperature). Slides were slightly fixed in 4% paraformaldehyde, washed and mounted with ProLong (Invitrogen). To localize ECE-1 in HEK cells, HEK-ECE-1c-GFP cells were fixed in paraformaldehyde and incubated with primary antibody to ECE-1 (goat antibody to human ECE-1, 1:200, overnight, 4°C). Cells were washed, incubated with secondary antibody conjugated to Rhodamine Red-X (1:500, 1 h, room temperature), and mounted.

Immunofluorescence in tissue sections

Rats were killed with sodium pentobarbital (200 mg·kg^{-1}, i.p.). Dorsal skin was incubated in 30% sucrose in PBS (24 h, 4°C), embedded in optimal cutting temperature (OCT) compound (Miles, Elkhart, IN), and sectioned at 10 μm. Slides were fixed in chilled methanol for 1 h and washed in PBS containing 5–10% normal donkey serum and 0.3% Triton X-100. Sequential sections were incubated with primary antibodies to ECE-1 (goat antibody to human ECE-1, 1:200), NK_1 receptor (#94168, 1:500) and the endothelial marker RECA-1 (1:500) (overnight; 4°C). Specificity of the NK_1 receptor antibody has been established (Grady *et al*., 1996). Sections were washed and incubated with secondary antibodies coupled to Alexa 568 or Alexa 488 (1:1000, 2 h, room temperature). Slides were washed and mounted in ProLong (Invitrogen).

ECE-1 antibody pre-absorption

Membranes were prepared from HEK cells transiently transfected with ECE-1c-GFP or empty vector (control) (Padilla *et al*., 2007), and integral membrane proteins were solubilized in 1% Triton X-100 in PBS. Diluted ECE-1 antibody (1:200, PBS, 5% normal donkey serum, 0.1% Triton X-100) was pre-incubated with membrane proteins (10 mg·mL^{-1}, overnight, 4°C). Antibodies were cleared by centrifugation (16 000× *g*, 5 min, 4°C) before use.

Confocal microscopy

Cells and tissues were observed by using a Zeiss Axiovert with a Zeiss 510 Meta confocal microscope with Apo Chromat 20× (NA 0.8) and Zeiss Plan Apo 100× (NA 1.4) objectives. Images were collected at zoom of 1–2, an iris of <2.5 μm, and typically 5–10 optical sections were taken at intervals of 0.5–1.5 μm. Images (single optical sections are shown) were coloured and processed to adjust contrast and brightness using Adobe Photoshop CS (Adobe Systems, Mountain View, CA, USA).

SP-induced plasma extravasation

Rats were anaesthetized with sodium pentobarbital (50 mg·kg^{-1} i.p.). SP (5 μg·kg^{-1} of a 5 μg·mL^{-1} solution) or physiological saline (control) were injected into the tail vein. To assess desensitization and re-sensitization of SP-induced plasma extravasation, rats received a second dose of SP (5 μg·kg^{-1}, i.v.) at 10–480 min after the first injection, followed immediately with Evans blue (30 mg·kg^{-1} in saline, i.v.). At 5 min after injection of Evans blue, rats were transcardially perfused with 100 mmol·L^{-1} PBS pH 7.4, and tissues were collected and weighed. Tissue was divided and half of the tissue was dried (48 h, 60°C) and then reweighed. Evans blue was extracted from the remaining tissue by incubation in formamide (48 h, room temperature). Evans blue was quantified by reading its absorbance at 620 nm using a standard curve (0.5–10 μg·mL^{-1}, Evans blue in formamide). Extravasation was expressed as μg of Evans blue per milligram of dry weight to correct for tissue oedema and then expressed as a percentage of the values obtained from vehicle-treated rats for each time point. To examine the role of ECE-1 in re-sensitization of NK_1 receptor-dependent plasma extravasation, rats were pre-treated with SM-19712 (20 mg·kg^{-1} of a 12.5 mg·mL^{-1} solution, p.o.), 1 h before and 3 h after the initial SP challenge (Umekawa *et al*., 2000). Rats received a second dose of SP at 480 min after the initial challenge, and Evans blue extravasation was measured. To determine whether ET-1 contributes to SP-induced plasma extravasation, rats were pre-treated with PD145065 (1 mg·kg^{-1} of a 1 mg·mL^{-1} solution, i.v.), an antagonist of ET_A and ET_B receptors (Doherty *et al*., 1993), or vehicle (control), 5 min before SP injection (5 μg·kg^{-1}, i.v.), and Evans blue extravasation was measured.

Statistical analysis

Results are expressed as mean ± SEM from $n \geq 3$ experiments. Results are compared by Student's *t*-test for two comparisons with $P < 0.05$ considered significant.

Reagents

Antibodies were from the following sources: rabbit antibody to the N-terminus of human ECE-1 (#52-6497, Invitrogen, Carlsbad, CA), goat anti-human ECE-1 (R&D System, Inc., Minneapolis, MN), rabbit anti-NK_1 receptor (#94168, Grady *et al*., 1996), mouse anti-early endosomal antigen 1 (EEA1) (BD Transduction Laboratories, Lexington, KY), mouse anti-RECA-1, a cell surface antigen expressed by all rat endothelial cells (Serotec Inc., Raleigh, NC), goat anti-mouse or anti-rabbit IgG coupled to fluorescein isothiocyanate or Cyanine 5 (Jackson ImmunoResearch, West Grove, PA), and donkey anti-goat or rabbit IgG coupled to Alexa 568, Alexa 488

or Rhodamine Red-X (Invitrogen). The ECE-1 inhibitor, SM-19712 (4-chloro-N-[[(4-cyano-3-methyl-1-phenyl-1H-pyrazol-5-yl)amino]carbonyl] benzenesulphonamide, monosodium salt) (Umekawa *et al.*, 2000), hydrocortisone acetate and N6,2′-O-dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt were from Sigma-Aldrich (St. Louis, MO). The non-selective ET_A and ET_B receptor antagonist PD145065 (N-acetyl-α-[10,11-dihydro-5H-dibenzo[a,d]cycloheptadien-5-yl]-D-Gly-Leu-Asp-Ile-Ile-Trp) (Doherty *et al.*, 1993) and human epidermal growth factor were from Calbiochem (San Diego, CA). SP and Sar^9,$Met(O_2)^{11}$-SP (SMSP), a NK_1 receptor-selective agonist (Drapeau *et al.*, 1987), were from Bachem (Torrance, CA). MCDB131 medium and Alexa 546 labelling kit were from Invitrogen. SMSP was labelled with Alexa 546 as described (Roosterman *et al.*, 2007).

Results

ECE-1 is endogenously expressed in HMEC-1 cells

We examined expression of ECE-1 in untransfected HMEC-1, a human microvascular endothelial cell line, using RT-PCR with primers specific for ECE-1 isoforms, and localized ECE-1 by immunofluorescence using an antibody that interacts with ECE-1b and ECE-1d (Padilla *et al.*, 2007; Roosterman *et al.*, 2007). We amplified mRNA encoding each of the ECE-1 isoforms (ECE-1a-d) from HMEC-1 and confirmed identity by sequencing (Figure 1A). Immunoreactive ECE-1b/d was detected in cytoplasmic vesicles of HMEC-1 (Figure 1B). Thus, HMEC-1 cells express all ECE-1 isoforms and ECE-1b/d are in endosomes.

Alexa-SMSP traffics to early endosomes containing ECE-1 in HMEC-1-NK_1 receptor cells

We examined SP signalling and trafficking in HMEC-1. SP (10 nmol·L^{-1}) failed to increase $[Ca^{2+}]_i$ in HMEC-1, suggesting that these cells do not express functional NK_1 receptors at detectable levels (Figure 1C). To facilitate detection, we over-expressed the NK_1 receptors in these cells. SP induced a prompt increase in $[Ca^{2+}]_i$ in cells transfected with NK_1 receptors (Figure 1C), confirming expression of a functional receptor. We determined whether Alexa-SMSP traffics to endosomes containing ECE-1 in these endothelial cells. Alexa-SMSP bound to the plasma membrane at 4°C (not shown), and after washing and warming to 37°C for 30 min Alexa-SMSP was prominently co-localized in endosomes with ECE-1 and EEA1 (Figure 2A, arrows). Thus, Alexa-SMSP traffics to early endosomes containing ECE-1 isoforms in NK_1 receptor-expressing endothelial cells.

ECE-1 regulates re-sensitization of SP signalling in HMEC-1-NK_1 receptor cells

To determine if ECE-1 regulates re-sensitization of NK_1 receptor signalling in HMEC-1-NK_1 receptor cells, as it does in other cell types (Roosterman *et al.*, 2007), we examined the effect of ECE-1 inhibition on re-sensitization of SP-induced Ca^{2+} sig-

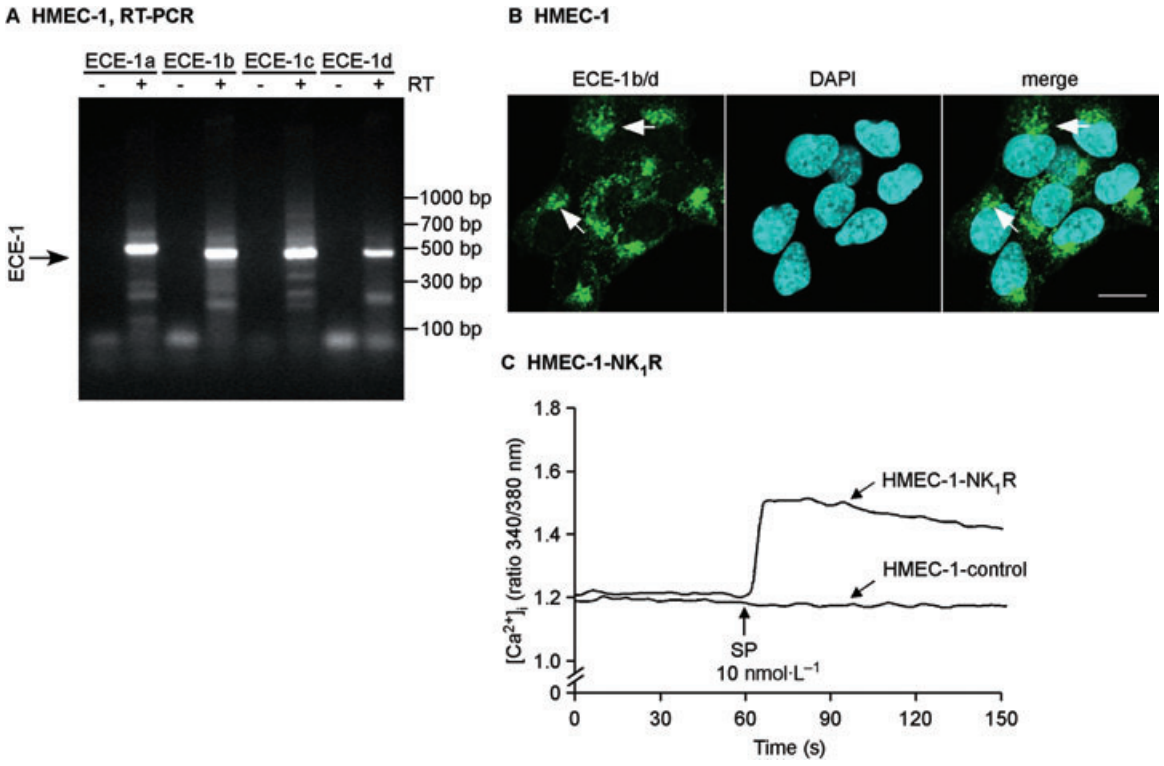

Figure 1 Expression of ECE-1 isoforms and NK_1 receptors (NK_1R) in HMEC-1 cells. (A) Amplification of mRNA transcripts encoding ECE-1a, -1b, -1c and -1d in HMEC-1 cells. (B) Detection of endogenous ECE-1 in HMEC-1 cells by immunofluorescence and confocal microscopy. Immunoreactive ECE-1b/d is localized to cytoplasmic vesicles (arrows). Scale bar, 10 μm. (C) SP-induced Ca^{2+} signalling in untransfected and NK_1 receptor transfected HMEC-1. SP (10 nmol·L^{-1}) increased $[Ca^{2+}]_i$ only in cells overexpressing NK_1 receptors.

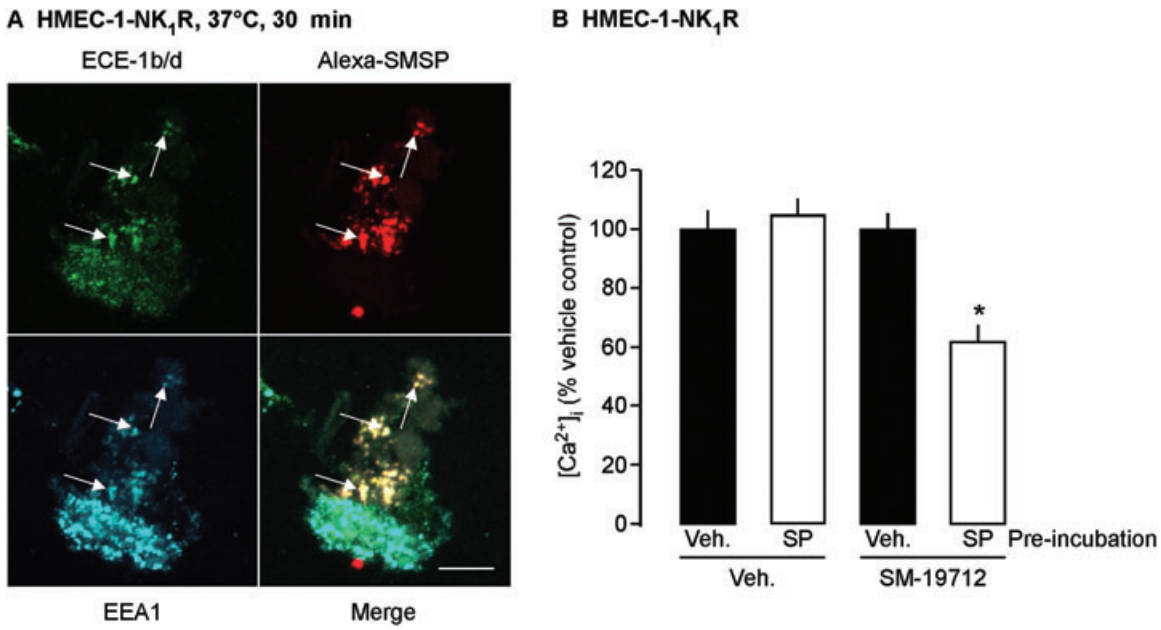

Figure 2 Internalized Alexa-SMSP co-localizes with ECE-1 in early endosomes and ECE-1 regulates re-sensitization of NK_1 receptor signalling in HMEC-1 cells. (A) HMEC-1-NK_1 receptor cells were incubated with Alexa-SMSP on ice for 2 h, washed and then warmed to 37°C for 30 min. Alexa-SMSP co-localized with immunoreactive ECE-1b/d and the early endosomal antigen 1, EEA1 (arrows). Scale bar, 10 μm. (B) Re-sensitization of SP-induced Ca^{2+} signalling. HMEC-1-NK_1 receptor cells were treated with SM-19712 or vehicle (veh., control). Cells were pre-incubated with SP (10 nmol·L^{-1}, 10 min) or vehicle (control), washed and challenged with SP (10 nmol·L^{-1}) after 30 min. Bars represent the increase in $[Ca^{2+}]_i$ in response to the SP challenge in cells pre-incubated with SP (open bars) or vehicle (closed bars). Results are expressed as % response in vehicle-treated cells (100%). SM-19712 inhibited re-sensitization in HMEC-1-NK_1 receptor cells (*$P < 0.05$ compared with vehicle).

nalling. Cells were incubated with SP for 10 min to desensitize the NK_1 receptors (Roosterman *et al.*, 2007), or vehicle (control). Cells were washed and recovered in SP-free medium for 30 min to allow re-sensitization to proceed. In cells not treated with the ECE-1 inhibitor, re-sensitization was complete within 30 min (Figure 2B, 105 ± 6% re-sensitization). In contrast, in cells treated with the ECE-1 inhibitor SM-19712, re-sensitization was strongly inhibited (Figure 2B, 62 ± 6% re-sensitization). Thus, ECE-1 regulates the re-sensitization of SP-induced Ca^{2+} signalling in endothelial cells that express NK_1 receptors.

ECE-1 co-localizes with NK_1 receptors in dermal endothelial cells

To determine if NK_1 receptors and ECE-1 are expressed in endothelial cells in intact tissues, we used immunofluorescence and confocal microscopy. We assessed specificity of the goat anti-human ECE-1 antibody by pre-absorption of the antibody with membrane extracts from HEK cells overexpressing ECE-1c-GFP or from non-transfected cells. Pre-absorbed antibodies were used to localize ECE-1c in HEK cells expressing ECE-1c-GFP. ECE-1c-GFP was detected at the plasma membrane and in endosomes of HEK-ECE-1c-GFP cells (Figure 3A), a result that is consistent with previous reports (Padilla *et al.*, 2007; Roosterman *et al.*, 2007). Antibody that was pre-incubated with membrane proteins from untransfected cells detected immunoreactive ECE-1 at the plasma membranes and in endosomes, and co-localized with ECE-1c-GFP (Figure 3A, upper panels). Pre-incubation with membrane proteins from cells expressing ECE-1c-GFP completely abolished staining (Figure 3A, lower panels). We then used these antibodies to localize ECE-1 in sections of rat dorsal skin. ECE-1 antibody that was pre-incubated with membrane proteins from untransfected cells strongly stained dermal blood vessels (Figure 3B). In contrast, pre-incubation with membrane proteins from cells expressing ECE-1c-GFP abolished staining (Figure 3B). Moreover, omission of primary antibodies dramatically decreased immunoreactivity of both ECE-1 and NK_1 receptors (not shown). Together these results suggest that the goat anti-human ECE-1 antibody specifically recognizes ECE-1 in rat tissue.

To determine if NK_1 receptors and ECE-1 are expressed in the same endothelial cells, we localized the NK_1 receptors and ECE-1 in serial sections of rat dorsal skin. Immunoreactive ECE-1 and NK_1 receptors were partially co-localized with the immunoreactive endothelial marker, RECA, and are thus present in endothelial cells of the skin (Figure 3C). Immunoreactive ECE-1 and NK_1 receptors were detected in the same endothelial cells, and also in smooth muscle. Thus, ECE-1 is appropriately localized to regulate SP signalling and the NK_1 receptor in endothelial cells in the skin.

ECE-1 inhibition prevents re-sensitization of SP-induced inflammatory plasma extravasation

Substance P induces endocytosis and recycling of the NK_1 receptor in endothelial cells of post-capillary venules, which correlates with desensitization and re-sensitization of SP-induced plasma extravasation (Bowden *et al.*, 1994). To further investigate the role of ECE-1 in regulating SP-induced

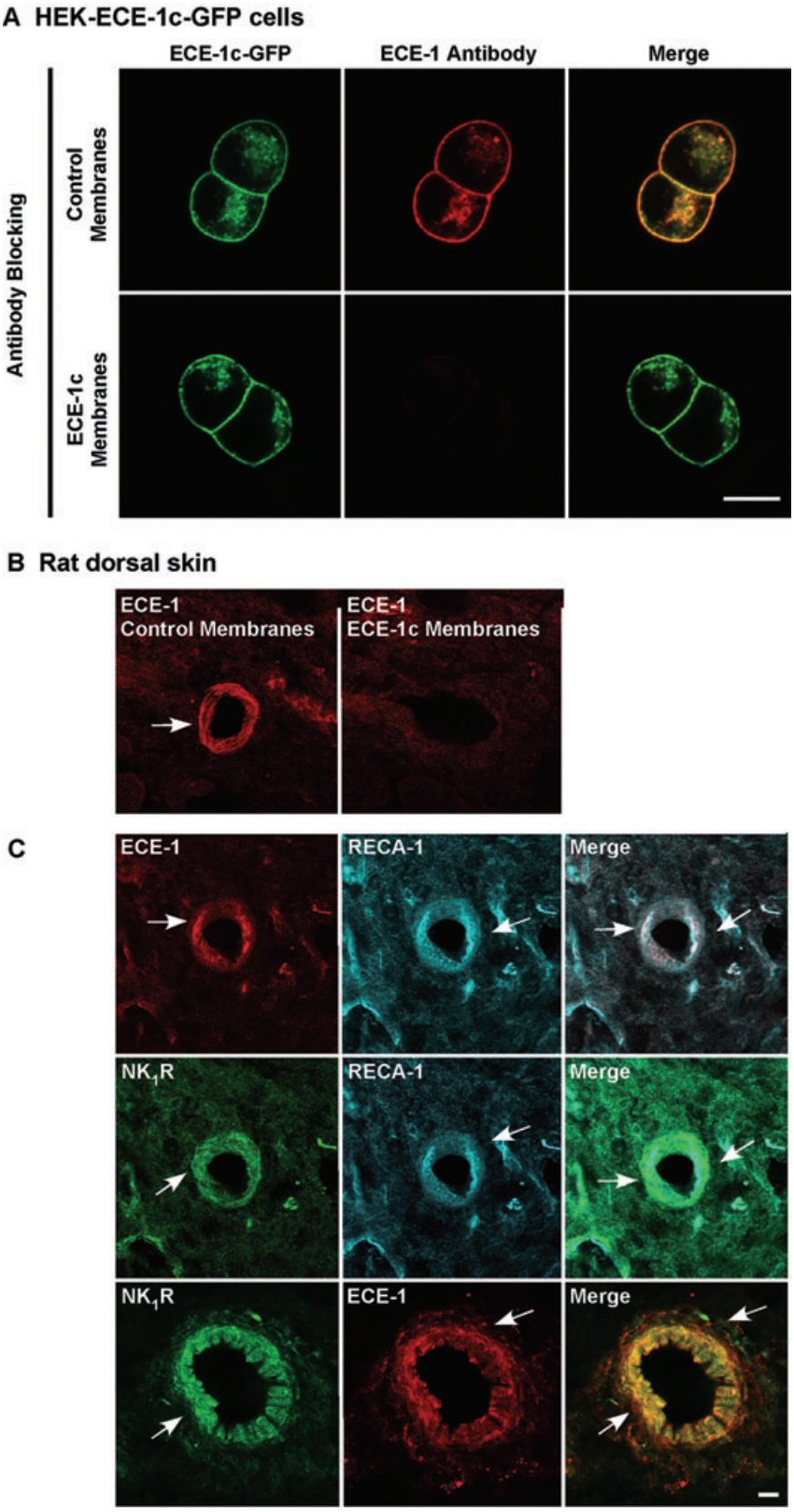

Figure 3 Co-localization of ECE-1 and NK_1 receptors in vessels of rat dorsal skin. (A) Localization of ECE-1c in HEK cells. HEK-ECE-1c-GFP cells were incubated with goat anti-human ECE-1c antibody pre-absorbed with membranes from HEK cells expressing empty vector (control) or membranes from cells overexpressing ECE-1c-GFP. In controls (upper panels), ECE-1c-GFP co-localized with immunoreactive ECE-1c at the plasma membrane and in endosomes. (B, C) Localization of ECE-1 and NK_1 receptors in sequential sections of the skin. (B) Pre-absorption with ECE-1c membranes abolished ECE-1 staining. (C) Co-localization of ECE-1 and NK_1 receptors with an endothelial cell marker, RECA-1. Both ECE-1 and NK_1 receptor immunoreactivity were detected in endothelial cells of rat dorsal skin. Scale bar (A), 10 μm; (B, C), 100 μm.

neurogenic inflammation, we examined the re-sensitization of SP-induced plasma extravasation in the urinary bladder, dorsal skin and ears of rats. To assess the time course of desensitization and re-sensitization of plasma extravasation, rats were injected with SP or saline (control), and then re-challenged with SP 10–480 min later. SP-induced extravasation of Evans blue was significantly reduced in dorsal skin when the interval between two injections of SP was 10 min (34 ± 5% of control) and reduced in the bladder (76 ± 25%) and the ears (56 ± 17%) (Figure 4A–C). This desensitization was still apparent when the second challenge of SP was 60 min later (bladder, 45 ± 9%; dorsal skin, 53 ± 19%; ears, 35 ± 11% of control) or 240 min later (bladder, 74.4.9 ± 14%; dorsal skin, 59.9 ± 15%; ears, 46.2 ± 8% of control). When the timing between the doses of SP was increased to 480 min, SP-induced extravasation of Evans blue had fully re-sensitized (bladder, 133 ± 52%; dorsal skin, 150 ± 30%; ears, 75 ± 15% of control). Thus, SP-induced plasma extravasation undergoes rapid desensitization and slow re-sensitization.

To examine whether ECE-1 regulates re-sensitization of SP-induced plasma extravasation, we examined re-sensitization at 480 min in animals treated with the ECE-1 inhibitor, SM-19712. SM-19712 did not affect the magnitude of extravasation after a single challenge with SP (not shown), suggesting that ECE-1 does not degrade SP in the extracellular fluid to attenuate its pro-inflammatory actions. However, SM-19712 significantly reduced re-sensitization of SP-induced Evans blue extravasation in the bladder (30 ± 4% of vehicle control), dorsal skin (41 ± 7%) and ears (48 ± 8%) (Figure 5A–C). Thus, ECE-1 promotes re-sensitization of SP-induced inflammation in the urinary bladder, dorsal skin and ears of the rat.

The ET-1 can induce plasma extravasation (Brandli *et al*., 1996). To exclude the possibility that the effects of the ECE-1 inhibitor SM-19712 on SP-induced plasma extravasation may be mediated by altered generation of ET-1, we examined the effects of PD145065, an antagonist of ET_A and ET_B receptors (Doherty *et al*., 1993), or vehicle (control) on SP-induced plasma extravasation. Compared with vehicle, PD145065 had no significant effect on SP-induced plasma extravasation in the bladder (115 ± 18% of vehicle control), dorsal skin (73 ± 10%) and ears (84 ± 13%) (Figure 6). Thus, ET-1 does not contribute to SP-induced plasma extravasation, and the effects of SM-19712 on re-sensitization of SP-induced plasma extravasation are unlikely to be due to reduced formation of ET-1.

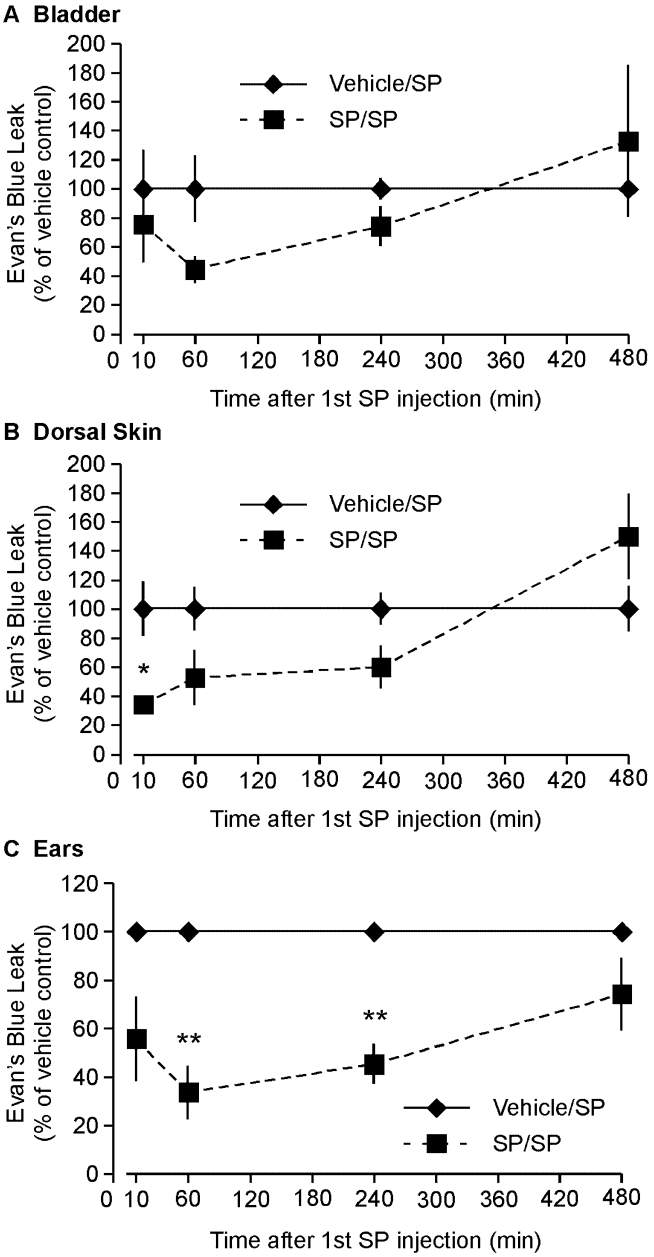

Figure 4 Time course of desensitization and re-sensitization of SP-induced extravasation of Evans blue. Rats were injected with SP (5 μg·kg^{-1} i.v.) or saline (control), followed by a second injection of SP (5 μg·kg^{-1} i.v.) 10–480 min later. Evans blue extravasation in response to the second injection of SP was determined in the urinary bladder (A), dorsal skin (B) and ears (C). When the interval between SP injections was 10 min, SP-induced Evans blue leak was significantly reduced in dorsal skin (*$P < 0.05$), and reduced in the bladder and in the ears. Desensitization was still apparent when the second challenge with SP was 60 or 240 min later in the ears (**$P < 0.01$), bladder and dorsal skin. Re-sensitization was complete after 480 min. Results are expressed as a percentage of control.

Discussion and conclusions

Our results show that ECE-1 induces re-sensitization of NK_1 receptors in microvascular endothelial cells and thereby promotes re-sensitization of the pro-inflammatory effects of SP in multiple tissues. We found that human microvascular endothelial cells endogenously expressed all ECE-1 isoforms, and that Alexa-SMSP, a full and selective NK_1 receptor agonist (Drapeau *et al*., 1987), internalized and trafficked to early endosomes containing ECE-1. A membrane permeant and selective ECE-1 inhibitor, SM-19712 (Umekawa *et al*., 2000), strongly inhibited re-sensitization of SP-induced, NK_1 receptor-mediated Ca^{2+} signalling in human microvascular endothelial cells. ECE-1 also co-localized with the NK_1 receptor in endothelial cells of the rat skin, and SM-19712 inhibited re-sensitization of SP-induced plasma extravasation in multiple tissues that are established sites of neurogenic inflammation. Together these results support a more general

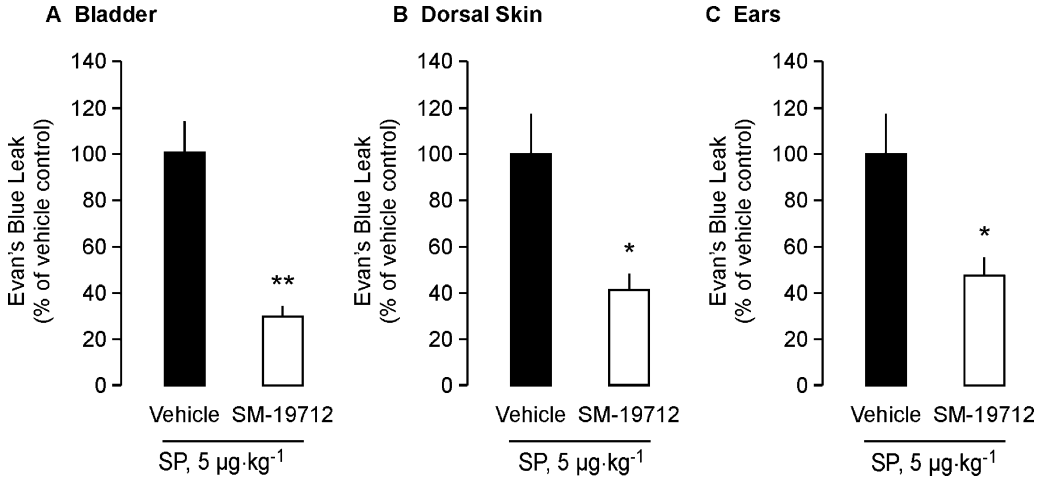

Figure 5 Effect of ECE-1 inhibition on re-sensitization of SP-induced Evans blue extravasation. Rats were treated with SM-19712 (20 mg·kg^{-1}, p.o. every 4 h). Rats were injected with SP (5 µg·kg^{-1} i.v.), followed by a second injection of SP (5 µg·kg^{-1} i.v.) 480 min later. Evans blue extravasation in response to the second injection of SP was determined in the urinary bladder (A), dorsal skin (B) and ears (C). SM-19712 significantly reduced Evans blue extravasation in response to the second injection of SP in the bladder (**$P < 0.01$), dorsal skin (*$P < 0.05$) and ears (*$P < 0.05$). Results are expressed as percentage of control.

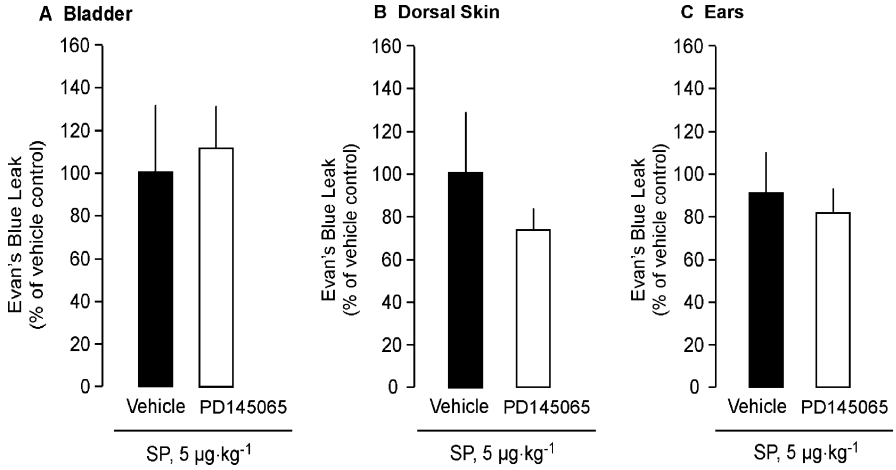

Figure 6 Effect of antagonism of ET_A and ET_B receptors on SP-induced Evans blue extravasation. Rats were pre-treated with PD145065 (1 mg·kg^{-1}) or vehicle (control). After 5 min, rats were injected with SP (5 µg·kg^{-1} i.v.), followed immediately by Evans blue. Evans blue extravasation was measured after 5 min in the urinary bladder (A), dorsal skin (B) and ears (C).

role for ECE-1 in addition to activating big ET within endothelial cells (Xu *et al.*, 1994; Turner and Murphy, 1996). Since ECE is present in invertebrates and has been highly conserved during evolution (Macours and Hens, 2004), it is likely to have additional major and unexplored biological roles. Although intracellular ECE-1 accounts for 85% of the total ECE-1 activity of endothelial cells (Russell and Davenport, 1999), the function of endosomal ECE-1 is not fully understood. We recently reported that ECE-1 can degrade neuropeptides such as SP and CGRP in acidified early endosomes to control recycling and re-sensitization of their receptors (Padilla *et al.*, 2007; Roosterman *et al.*, 2007), but the functional significance of this regulation was unclear. The present results suggest that this mechanism promotes re-sensitization of the pro-inflammatory effects of SP in the endothelium of several tissues, supporting a widespread and general role for ECE-1 in controlling neurogenic inflammation.

In the current study, we examined whether ECE-1 promotes re-sensitization of NK_1 receptor signalling in endothelial cells by examining the effect of ECE-1 inhibition on re-sensitization of SP-induced Ca^{2+} signalling in a human microvascular endothelial cell line. These cells naturally expressed all isoforms of ECE-1. Although microvascular endothelial cells from the dermis (HDMEC-1) endogenously express NK_1 receptors (Quinlan *et al.*, 1998), we did not detect responses to SP in HMEC-1, and therefore we over-expressed the NK_1 receptor to allow investigation of SP trafficking and signalling. We observed that SM-19712 strongly inhibited re-sensitization of the NK_1 receptor in HMEC-1-

NK_1 receptor cells. These results are consistent with our findings in other cell types (human and rat kidney epithelial cells and neuroblastoma cells) that ECE-1 inhibition and knock-down impedes recycling and re-sensitization of receptors for SP and CGRP, whereas ECE-1 overexpression has the opposite effects (Padilla *et al.*, 2007; Roosterman *et al.*, 2007). We also observed that SMSP trafficked from the plasma membrane to early endosomes containing endogenous ECE-1 in microvascular endothelial cells, and it is likely that endosomal ECE-1 degrades SP in endothelial cells. Indeed, ECE-1 degrades several neuropeptides at an acidic pH optimum similar to that of early endosomes, and ECE-1 mediates degradation of endocytosed SP, CGRP and somatostatin (Johnson *et al.*, 1999; Padilla *et al.*, 2007; Roosterman *et al.*, 2007; 2008). Endocytosed SP, CGRP and somatostatin also traffic to endosomes containing all ECE-1 isoforms in several different cell types (Padilla *et al.*, 2007; Roosterman *et al.*, 2007; 2008). The mechanism by which ECE-1 promotes recycling of G-protein coupled receptors from endosomes and subsequent re-sensitization of signalling appears to be common for receptor for peptides that are ECE-1 substrates at endosomal pH, and for those receptors that exhibit sustained interactions with β-arrestins in endosomes (Padilla *et al.*, 2007; Roosterman *et al.*, 2007; 2008). By degrading neuropeptides such as SP, CGRP and somatostatin in endosomes, ECE-1 disrupts the peptide-receptor-β-arrestin complex, allowing the receptor, freed from β-arrestins, to recycle and re-sensitize the cell. Whether this mechanism controls the recycling and re-sensitization of other receptors in endothelial cells, in addition to the NK_1 receptors, remains to be determined.

We further characterized the role of endosomal ECE-1 in endothelial cells by examining the effects of an ECE-1 inhibitor on the pro-inflammatory effects of SP *in vivo*. Numerous noxious and pro-inflammatory stimuli induce release of SP and CGRP from a subpopulation of primary spinal afferent nerve fibres in peripheral tissues. SP acts on the NK_1 receptors of endothelial cells of post-capillary venules to promote gap formation and the extravasation of plasma proteins, whereas CGRP causes arteriolar vasodilatation, which together comprise neurogenic inflammation (McDonald *et al.*, 1996). We observed that immunoreactive ECE-1 and the NK_1 receptor co-localized in endothelial cells and vascular smooth muscle of the skin. Our results are consistent with previous reports showing localization of the NK_1 receptor in endothelial cells of post-capillary venules (Bowden *et al.*, 1996), and with the localization of ECE-1 to endothelial cell lines (Hunter and Turner, 2006) as well as in endothelial and smooth muscle cells of venules and arterioles (Korth *et al.*, 1999). SP stimulated the extravasation of plasma proteins in the urinary bladder, skin and ears, and this response rapidly desensitized when the interval between repeated injections of SP was 10 min, and slowly re-sensitized after 480 min. The time courses of these processes reflect the endocytosis and recycling of NK_1 receptors in post-capillary endothelial cells (Bowden *et al.*, 1994). The selective ECE-1 inhibitor SM-19712 significantly inhibited re-sensitization of SP-induced plasma extravasation in the urinary bladder, skin and ears, consistent with its effects in the mouse skin (Roosterman *et al.*, 2007). Thus, ECE-1 in endothelial cells acts to promote the recycling and re-sensitization of the NK_1 receptors. SM-19712 did not affect the magnitude of extravasation after a single challenge with SP (not shown), suggesting that, unlike neprilysin (Lu *et al.*, 1997; Sturiale *et al.*, 1999), ECE-1 does not degrade SP in the extracellular fluid to attenuate its pro-inflammatory actions.

ET-1 can promote inflammation by enhancing microvascular permeability (Brandli *et al.*, 1996). We observed that PD145065, an antagonist of ET_A and ET_B receptors (Doherty *et al.*, 1993), did not affect SP-induced plasma extravasation in any tissue examined. These results indicate that the effects of the ECE-1 inhibitor SM-19712 on SP-induced plasma extravasation are not due to reduced generation of ET-1, but are more likely to be due to reduced degradation of SP in endosomes and consequent inhibition of NK_1 receptor recycling and re-sensitization.

Whether ECE-1 regulates other effects of SP on endothelial cells remains to be determined. In addition to promoting plasma extravasation, SP acts on endothelial cells to cause expression of adhesion molecules (Quinlan *et al.*, 1998) infiltration of granulocytes (Smith *et al.*, 1993) and nitric oxide-dependent vasodilatation (Hall and Brain, 1994). These effects are mediated by the NK_1 receptor and could thus be controlled by ECE-1. ECE-1 may also regulate the pro-inflammatory actions of other neuropeptides that mediate neurogenic inflammation. ECE-1 promotes recycling and re-sensitization of CGRP receptors (Padilla *et al.*, 2007), but whether this mechanism induces re-sensitization of the pro-inflammatory effects of CGRP remains to be determined. It is also unknown whether ECE-1 controls re-sensitization of other effects of SP, such as pain transmission. However, given its widespread expression by multiple cell types, it is likely that ECE-1 has additional biological roles.

In conclusion, we report a novel biological role of ECE-1 in promoting NK_1 receptor trafficking and recycling in microvascular endothelial cells, to control the pro-inflammatory effects of SP. Inhibitors of endosomal ECE-1, by attenuating re-sensitization of receptors for neuropeptides such as SP and CGRP, may suppress neurogenic inflammation.

Acknowledgements

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Conflict of interest

None.

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