Characterisation of endothelin converting enzyme-1 shedding from endothelial cells

Sanjaya Kuruppu*, Shane Reeve, A. Ian Smith

Abstract The aim of this study was to determine if endothelin converting enzyme-1 (ECE-1) like other members of this metallopeptidase family undergoes ectodomain shedding. The release/shedding of catalytically active ECE-1 was measured by monitoring the fluorescence resulting from the cleavage of a specific quenched fluorescent substrate. Catalytically active ECE-1 was detected in the media of human umbilical vein endothelial cells, and was confirmed by mass spectrometry based assays. Specificity of cleavage was confirmed by using both narrow and broad specificity inhibitors. In conclusion we demonstrate and characterize for the first time, ECE-1 shedding from the surface of endothelial cells.

Keywords: Endothelin; ECE-1; Fluorescence; Ectodomain shedding; Mass spectrometry

1. Introduction

Since its discovery over 15 years ago, endothelin (ET) has attracted considerable scientific and clinical interest, reflecting its extremely potent vasoconstrictor effects [1]. ET is stored in an inactive form (BigET-1) and upon secretion is cleaved by a highly specific metallopeptidase called endothelin converting enzyme-1 (ECE-1) to liberate the 21 amino acid bioactive ET [2]. ECE-1 is a type II integral membrane metalloprotease with a zinc co-ordinating motif and the protease is expressed primarily on the plasma membrane of vascular endothelial cells. ECE-1 is highly specific and cleaves its substrate (BigET-1) between Trp21 and Val/Ile22. However, ECE-1 also cleaves bradykinin in vitro [3], which may indicate additional natural substrates for the enzyme.

Many cellular membrane proteins including enzymes are known to be cleaved by proteases to release the extracellular, often bioactive region in a process known as ‘ectodomain shedding’ [4]. This process can release growth factors, cytokines and other mediators from their membrane-bound precursors or result in the down-regulation of membrane-bound receptors [5]. This is therefore an important mechanism that regulates many physiological and pathophysiological events such as cell growth [6] and disease processes [6,7]. Recent studies from our and other laboratories have shown that the catalytically active ectodomain of angiotensin converting enzyme-2 (ACE2), a metallopeptidase related to ECE-1, is also shed from the cell surface in vitro [4,8] and that this process is further stimulated by the activation of protein kinase C (PKC) by phorbol 12-myristate 13-acetate (PMA) [4].

Many of the ectodomain cleavage events are mediated by Ca2+/Zn2+ dependent endopeptidases, matrix metalloproteinases (MMPs) and the closely related Zn-dependent ‘A disintegrin and metallopeptidases (ADAMs)’ [5,7,9–11]. The current understanding of the function of this family of metalloproteinases in ectodomain shedding is based on studies of ADAM-10 and ADAM-17. However, the contribution of other ADAMs is increasingly evident [5]. ADAM-17 also referred to as tumour necrosis factor-α converting enzyme (TACE) was the first sheddase to be isolated with a known physiological substrate. TACE is implicated in the release of L-selection [12], b-APP [13] and EGF receptor ligands [14] as well as ACE2 [4].

We hypothesized that the ectodomain of ECE-1 can also be released into the extracellular milieu by a regulated cleavage event. Proteolytic shedding of ECE-1 was measured by monitoring the cleavage of quenched fluorescent substrate (QFS) in conditioned media. A mass spectrometry approach involving BigET18–34 was used to confirm for the first time that ECE-1, endogenously expressed by human umbilical vein endothelial cells undergoes ectodomain shedding. Our data also suggest that ADAM family of metalloproteinases or MMPs are not involved in the ECE-1 ectodomain cleavage.

2. Methods

2.1. Cell culture

Human umbilical vein endothelial cells (HUVECs) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 8% (v/v) fetal bovine serum, 1% penicillin/streptomycin/glutamine and HAT supplement (1 vial/500 mL of media; Sigma), as mentioned previously [15].

2.2. Preparation of cell membranes

Confluent cells were washed twice with phosphate buffered saline and scrapped from petri dishes into 30 mM HEPES containing 20 mM CaCl2 (pH 7.4). Cells were lysed by sonication using a Sonifer
3.2. BigET and BigET<sub>18–34</sub> assay and mass spectrometry

The reaction mixture containing BigET<sub>18–34</sub> (MW 1907 Da) and conditioned media alone, as well as in the presence of inhibitors thiorphan/phosphoramidon was analysed by MALDI-ToF at the indicated time points. All MALDI spectra at time = 0 min confirmed the presence of BigET<sub>18–34</sub>, but neither the C-terminal cleavage product BigET<sub>22–34</sub> (MW 1380 Da), nor the N-terminal cleavage product BigET<sub>18–21</sub> (MW 1907 Da) was detected (data not shown). BigET<sub>32–34</sub> was detected in the mixture containing media alone, and in the presence of thiorphan after 2 and 5 h, respectively (Fig. 3a and b). Five hours of incubation failed to detect this product in the reaction mixture containing media and phosphoramidon (Fig. 3c). BigET<sub>18–21</sub> was not detected in any of the MALDI spectra generated.

The reaction mixture containing BigET and conditioned media alone, as well as in the presence of inhibitors thiorphan/phosphoramidon was also analysed by MALDI-ToF at time = 0, 1 and 5 h. Spectra at time = 0 confirmed
the presence of BigET, while endothelin-1 (ET-1) or the C-terminal cleavage product (BigET22–38; MW 1808) was not detected (Fig. 4a). BigET22–38 was detected 1 h after incubation with media alone (Fig. 4b). However, the presence of phosphoramidon in the reaction mixture appeared to significantly reduce the rate of BigET cleavage. This is indicated by the reduced intensity of the signal corresponding to BigET22–38 (Fig. 4c). In parallel experiments cleavage of BigET was examined in the presence of the NEP inhibitor thiorphan, where cleavage was comparable to that observed under control conditions (data not shown).

3.3. Nano-LC MSMS analysis

Following reverse phase separation of the reaction mixture consisting of BigET18–34 and conditioned media, the fractions containing BigET18–34 and BigET22–34 (m/z 1380) was analysed by tandem mass spectrometry. The amino acid sequence of the resulting peptide fragments and their corresponding molecular masses are listed in Tables 1 and 2. In addition to the m/z ratio obtained by mass spectrometry, the results of tandem mass spectrometry analysis provides further proof for cleavage of BigET18–34 by conditioned media between Trp21 and Val22.

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Fig. 1. The effect of (a) membrane proteins or (b) conditioned media alone (from HUVECs) and in the presence of thiorphan or phosphoramidon on the cleavage of QFS. *Significantly different from conditioned media in the presence of thiorphan; n = 3, one-way ANOVA.

Fig. 2. The effect of metalloprotease inhibitors, GM6001 and TAPI-1 on the cleavage of QFS by conditioned media (from HUVEC) alone and in the presence of thiorphan or phosphoramidon. *Significantly different to control media alone; two-way ANOVA; n = 3.
4. Discussion

Ectodomain shedding has emerged as an important mechanism which regulates the functions of various integral membrane proteins which include, adhesion molecules, cytokines, growth factors and their receptors [17–19]. Human umbilical vein endothelial cells are known to release endothelins [20] and endogenously express ECE-1 [21]. Therefore, these cells were chosen to study the mechanisms behind the shedding of the catalytically active ectodomain of ECE-1. However, the co-expression of closely related NEP enzyme by HUVECs added a degree of complication to the study, requiring the assays to be conducted in the presence of specific NEP inhibitor thiorphan. The NEP homologue NEP-2, has high sequence identity with NEP and is known to be localized only in the central nervous system and testis, in this context, phosphoramidon is more effective than thiorphan in inhibiting NEP-2 [22].

The conditioned media and cell membrane preparations were initially assayed for ECE-1 activity based on their ability to cleave QFS. The amino acid sequence of QFS used in this study is based on that of bradykinin, another known substrate for ECE-1 [3,23]. Despite it also being a substrate for NEP, QFS is hydrolysed 10-fold more efficiently by ECE-1 ($K_{cat}/K_m$ value $1.9 \times 10^7$ M$^{-1}$ S$^{-1}$ [16]) making this substrate more selective for ECE-1. The amount of NEP in the conditioned media and membrane preparations, as evidenced by the re-
duced rate of fluorescence in the presence of thiorphan, varied considerably amongst cell passages. However, the presence of ECE-1 was always confirmed upon addition of phosphoramidon, a dual ECE-1 and NEP inhibitor which significantly reduced the rate of fluorescence compared to thiorphan.

It has been widely reported that various cell surface proteins are shed upon stimulation by PMA which activates PKC, thus triggering a metalloproteinase dependent ectodomain shedding machinery [24,25]. This pathway is described as ‘inducible shedding’ and is known to be highly conserved amongst multiple cell types [17]. The results of this study indicated that ECE-1 is only released constitutively and that PMA has no effect on the shedding process. Although previous studies indicate that ECE-1 migrates to the cell surface upon stimulation by PMA [26], this was not confirmed by the results of this study.

Members of the metalloproteinase super family have been shown to be responsible for the cleavage of a majority of shed proteins [27]. Therefore, to determine the involvement of these proteins in the cleavage of ECE-1, a broad spectrum metalloproteinase inhibitor GM6001 was added to the cells. Identification of the specific metalloproteinase responsible for ECE-1 shedding was undertaken based on reports that ADAM-10 and ADAM-17/TACE, have been implicated in the constitutive and PMA induced shedding of a number of cell surface expressed molecules [17,27,28]. Most MMPs are blocked by the TIMPs 1, 2 and 3, whereas ADAM-10 is inhibited by TIMP-1 and TACE activity is sensitive to TAPI-1 and TIMP-3 [29–31]. Results of experiments using TIMP-1 and TIMP-3 ruled out MMP and ADAM-10, mediated shedding of ECE-1. TAPI-1 reduced the rate of fluorescence to a level similar to that observed in the presence of GM6001 suggesting a role for TACE in the shedding process. Although a decrease in the rate of fluorescence in response to TIMP-3 (a known TACE inhibitor) was expected, this was not observed. However, the lack of a significant difference in the fluorescence induced by media treated with GM6001/TAPI-1 (+thiorphan), compared to control media (+thiorphan) indicates that the inhibitors blocked NEP release as opposed to ECE-1 release. Since neither inhibitor completely abrogated NEP or ECE-1 release, another mechanism(s) may regulate their release. Such mechanisms have been postulated in relation to the release of other membrane proteins such as Axl [17].

Since the QFS used in this study can also be cleaved by NEP, we obtained further proof for the presence of ECE-1 in the media by using ECE-1 specific natural substrate BigET 18–34, which is known to undergo 60% cleavage by ECE-1 [32]. Analysis by liquid chromatography indicated the cleavage of BigET 18–34 by cell membrane preparations as well as conditioned media, as evidenced by a decreasing substrate peak area with increasing time. However, the cleavage products appeared to undergo secondary degradation, and were below the level of detection by liquid chromatography. Therefore, we undertook a mass spectrometry approach to determine the identity of the ECE-1 mediated cleavage products of BigET 18–34. The expected ECE-1 mediated C-terminal cleavage product was confirmed by mass as well as sequence, and further, it was inhibited by phosphoramidon but not thiorphan. This therefore clearly demonstrates the presence of ECE-1 in the conditioned media. Additional evidence was obtained by examining the cleavage of the natural substrate BigET as well by conditioned media. As observed with BigET 18–34, only the C-terminal cleavage product BigET 22–34 was observed. The cleavage of BigET was inhibited by phosphoramidon and not thiorphan, thus given that cleavage of BigET at this site is highly ECE specific, these data confirm ECE-1 mediated cleavage.

It is not known whether the surface of HUVECs express NEP-2. A comparison of the catalytic activities and substrate specificities of NEP and NEP-2 has been undertaken by Rose et al. [33]. The results presented would strongly suggest that NEP-2 is unlikely to be involved in the cleavage of the substrates used in our study, particularly in the face of the high

### Table 1
Peptide fragments of BigET_{18–34}(MW 1907 Da)

<table>
<thead>
<tr>
<th>Actual mass (Da) as confirmed by MS/MS</th>
<th>Mass (Da) calculated based on amino acid sequence</th>
<th>Predicted amino acid sequence</th>
<th>Amino acid sequence confirmed by MS/MS</th>
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<td>364</td>
<td>381</td>
<td>PEH</td>
<td>PEH</td>
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<tr>
<td>562</td>
<td>579</td>
<td>PEHV; EHVVP</td>
<td>PEHV; EHVVP</td>
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<td>TPEHV</td>
<td>TPEHV</td>
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<td>857</td>
<td>IWVTPE1</td>
<td>IWVTPE1</td>
</tr>
<tr>
<td>1066</td>
<td>1112</td>
<td>NTPEHVVPY</td>
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### Table 2
Peptide fragments of BigET_{22–34}(MW 1380 Da)

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concentration of thiorphan used (100 μM) and the very much greater cleavage efficiency of bradykinin by NEP compared to NEP-2.

In conclusion, HUVECs endogenously express ECE-1 as well as NEP. Our results demonstrate for the first time that ECE-1 is shed from the surface of HUVECs via MMP or ADAM independent pathway, and is not stimulated by the activation of PKC. The release of NEP occurs to a lesser extent, and appears to be mediated by TACE. The results of this study highlight the potential for the use of circulating ECE-1 as a diagnostic or prognostic marker of pathological conditions such as cardiovascular diseases.

References