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ScienceDirect
Analytical Biochemistry 363 (2007) 255–262
ANALYTICAL BIOCHEMISTRY
www.elsevier.com/locate/yabio

Determination of angiotensin I-converting enzyme activity in cell culture using fluorescence resonance energy transfer peptides

R.A. Sabatini [a], P.A. Bersanetti [a], S.L. Farias [a], L. Juliano [a], M.A. Juliano [a], D.E. Casarini [b], A.K. Carmona [a], A.C.M. Paiva [a], J.B. Pesquero [a,*]

[a] *Department of Biophysics, Universidade Federal de São Paulo-Escola Paulista de Medicina, São Paulo, Brazil*
[b] *Department of Medicine, Division of Nephrology, Universidade Federal de São Paulo-Escola Paulista de Medicina, São Paulo, Brazil*

Received 5 December 2006
Available online 19 January 2007

Abstract

An assay using fluorescence resonance energy transfer peptides was developed to assess angiotensin I-converting enzyme (ACE) activity directly on the membrane of transfected Chinese hamster ovary cells (CHO) stably expressing the full-length somatic form of the enzyme. The advantage of the new method is the possibility of using selective substrates for the two active sites of the enzyme, namely Abz-FRK(Dnp)P-OH for somatic ACE, Abz-SDK(Dnp)P-OH for the N domain, and Abz-LFK(Dnp)-OH for the C domain. Hydrolysis of a peptide bond between the donor/acceptor pair (Abz/Dnp) generates detectable fluorescence, allowing quantitative measurement of the enzymatic activity. The kinetic parameter K_m for the hydrolysis of the three substrates by ACE in this system was also determined and the values are comparable to those obtained using the purified enzyme in solution. The specificity of the activity was demonstrated by the complete inhibition of the hydrolysis by the ACE inhibitor lisinopril. Therefore, the results presented in this work show for the first time that determination of ACE activity directly on the surface of intact CHO cells is feasible and that the method is reliable and sensitive. In conclusion, we describe a methodology that may represent a new tool for the assessment of ACE activity which will open the possibility to study protein interactions in cells in culture.

Keywords: Angiotensin I-converting enzyme; Cell culture; Fluorimetric assay; Fluorescence resonance energy transfer (FRET) peptides; Enzyme activity; CHO cells; Aorta smooth muscle cells (ASMC)

The surfaces of some cell types are endowed with proteolytic enzymes able to modulate the activity of membrane proteins or circulating regulatory peptides. Several of these enzymes, mostly metallopeptidases, are organized as ectoenzymes with their catalytic domains facing the extracellular region [1]. These ectopeptidases exist mainly as single membrane-spanning proteins of type I (C terminus intracellular) or type II (N terminus intracellular) topology or glycosyl-phosphatidylinositol-linked proteins. By far angiotensin I-converting enzyme (ACE)[1] is one of the most studied peptidases because of its broad physiological and pathological roles.

ACE (peptidyl dipeptidase A, kininase II, EC 3.4.15.1) is a zinc-dipeptidyl carboxypeptidase that cleaves the C-terminal dipeptide from angiotensin I to produce the potent vasopressor octapeptide angiotensin II [2] and inactivates bradykinin

[1] *Abbreviations used:* ACE, angiotensin I-converting enzyme; Abz, *ortho*-aminobenzoic acid; ASMC, aortic smooth muscle cells; Dnp, 2,4-dinitrophenyl; CHO, Chinese hamster ovary; FRET, fluorescence resonance energy transfer; MALDI-TOF, matrix-assisted laser desorption ionization time of flight; DMEM, Dulbecco's Modified Eagle's Medium; HBSS, Hank's balanced salt solution; AFU, arbitrary fluorescence units; TFA, trifluoroacetic acid; HUVEC, human vascular endothelial cells.

* Corresponding author. Fax: +55 11 5571 5780.
E-mail address: jbpesq@biofis.epm.br (J.B. Pesquero).

0003-2697/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2007.01.020

by the sequential removal of two C-terminal dipeptides [3,4]. Somatic ACE (150–180 kDa) consists of two homologous domains (N and C domains) within a single polypeptide sequence [5–7]. A smaller ACE isoform (90–100 kDa) is present in mature germinal cells and this testicular isoenzyme contains a single active site that corresponds to the C-terminal domain of the somatic enzyme with the exception of a short unique N-terminal sequence [8,9]. The somatic isoform is found in the plasma membrane of vascular endothelial cells, in epithelial cells of renal proximal tubules, in the gastrointestinal tract, in cardiac tissues, and in various regions of the brain [10,11]. In addition to these forms, Casarini et al [12,13] reported the presence of different naturally occurring ACE isoforms isolated from urine of normotensive (65 kDa) and hypertensive patients (65 and 90 kDa). Deddish et al. [14] also described a natural ACE N-domain isoform in human ileal fluid with molecular weight of 108 kDa.

Fluorescence resonance energy transfer (FRET) substrates hydrolyzed by both catalytic ACE domains and selective for each catalytic site, namely Abz-FRK(Dnp)P-OH for somatic ACE [15], Abz-SDK(Dnp)P-OH selective for N-domain [15], and Abz-LFK(Dnp)-OH selective for C-domain [16], have recently been described. These substrates containing a donor fluorophore (Abz) and an acceptor group (Dnp) have the advantage of high sensitivity and are important tools in enzyme detection assays even in low concentrations. Using Abz-FRK(Dnp)P-OH, Alves et al. [17] determined ACE activity in human plasma and in crude extracts of rat tissues. However, the analysis of ACE activity in cultured cells has so far not been described. Thus, the aim of this study was to determine ACE activity in a biologically active state using as substrates the FRET peptides. We explored this possibility in transfected Chinese hamster ovary cells (CHO) which stably express the full-length somatic form of this enzyme (CHO/ACE). In addition, we have also assessed ACE activity in aortic smooth muscle cells (ASMC) that express endogenous ACE and compared it with the activity of transfected CHO cells. The results indicated that the Abz/Dnp FRET peptides are useful for the measurement of ACE activity on the surface of cells in culture.

Materials and methods

Materials

Dulbecco´s modified Eagle´s medium (DMEM) and HAM´s F-12 medium were purchased from Gibco-BRL. Lisinopril was purchased from Sigma Chemical Co. All other chemicals were of analytical grade and commercially available.

Cell culture

Aortic smooth muscle cells were harvested from male Sprague–Dawley rats (3–6 months) kindly provided by Dr. Suma Imura Shimuta (UNIFESP, Brazil). Chinese hamster ovary cells were transfected either with a plasmid containing the whole coding region for the rat ACE (kindly supplied by Dr. François Alhenc-Gelas from the Institut National de la Santé et de la Recherche Médicale (Paris, France)), with the two intact functional domains carrying the neomycin-resistant gene, or with the vector alone (mock-transfected cells). Individual clones were selected in medium containing neomycin, isolated, propagated, and screened for ACE activity. CHO cells were cultured in HAM´s F-12 medium and ASMC were cultured in DMEM both supplemented with 10% fetal bovine serum (Gibco), 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate and incubated at 37 °C in a humidified 5% CO_2 in air. Medium was changed every 3–4 days and cells were passed every 6–8 days by harvesting with trypsin–EDTA.

Analysis of ACE expression by RT-PCR

The analysis of expression of ACE in transfected CHO cells was performed by RT-PCR. Total RNA was extracted from CHO/ACE cells using TRIzol reagent according to manufacturer's protocol. Integrity of the isolated RNA was assessed by separation on a 1% ethidium-bromide-stained agarose gel. Contaminant genomic DNA in RNA samples was avoided by treatment for 1 h at 37 °C with DNase I (1 U/2 μg RNA) in the presence of RNase-OUT-RNase Inhibitor (20 U) and 3 mM MgCl2. After the incubation period, samples were heated to 95 °C and immediately chilled on ice for DNase I denaturation. Reverse transcription was performed using 2 μg of total pure RNA, 200 U MMLV reverse transcriptase, 5 mM dithiothrectol, 50 ng Random Hexamers primers, 1x PCR buffer, 0.5 mM dNTPs, and 3 mM MgCl2. Reactions were submitted to the following protocol: 20 °C for 10 min, 42 °C for 45 min, 95 °C for 50 min, and 4 °C for 10 min. Resultant cDNA was then used for PCR as described next. Specific primers against rat ACE were synthesized (forward 5′- CTG CTA AGC AAC ATG AGC AG -3′, reverse 5′- GAT CCC CTG ATA CTT GGT TC -3′, Invitrogen Co., Carlsbad, CA, USA). RT-PCR was carried out using the MJ Research PTC200 (GMI, Inc., Ramsey, MN, USA). Cycling parameters were 4 min at 94 °C and 27 cycles of 50 s at 94 °C, 50 s at 60 °C, 1 min 20 s at 72 °C, and 7 min at 72 °C. The PCR products were analyzed by electrophoresis on a 1% agarose gel, visualized by ethidium bromide staining under UV illumination, and verified for their expected size (1060 bp according to various known ACE sequences).

ACE protein expression in transfected CHO cells

Protein expression was determined by Western blotting analysis. Cell extracts were separated by SDS–PAGE (7.5%) and transferred to a nitrocellulose membrane. The membranes were blocked for 30 min in TBS/T containing 3% nonfat milk and immunoblotted with an anti-ACE-