The secretases that cleave angiotensin converting enzyme and the amyloid precursor protein are distinct from tumour necrosis factor-α convertase

S. Parvathy[a], Eric H. Karran[b], Anthony J. Turner[a], Nigel M. Hooper[a,*]

[a]*School of Biochemistry and Molecular Biology, University of Leeds, Leeds LS2 9JT, UK*
[b]*Neurosciences Research, SmithKline Beecham Pharmaceuticals, Harlow CM19 5AW, UK*

Received 25 May 1998

Abstract Angiotensin converting enzyme (ACE) and the Alzheimer's amyloid precursor protein are cleaved from the membrane by zinc metalloproteinases termed ACE secretase and α-secretase, respectively. Tumour necrosis factor-α (TNF-α) convertase (ADAM 17) is a recently identified member of the adamalysin family of mammalian zinc metalloproteinases that is involved in the production of TNF-α and possibly in the cleavage of other membrane proteins. Using two different cell-free assays we were unable to detect significant cleavage and secretion of ACE by TNF-α convertase. In addition, there was a different effect of three hydroxamic acid-based inhibitors (batimastat, compound 1 and compound 4) towards TNF-α convertase as compared to ACE secretase and α-secretase. Thus TNF-α convertase would appear to be distinct from, but possibly related to, the secretases that cleave ACE and the amyloid precursor protein.

Key words: Zinc metalloproteinase; Angiotensin converting enzyme; Tumour necrosis factor-α convertase; Amyloid precursor protein; Hydroxamic acid inhibitor

1. Introduction

Numerous integral membrane proteins also exist in a soluble form as a result of a post-translational proteolytic cleavage event (reviewed in [1]). Examples of such proteins include angiotensin converting enzyme (ACE), the Alzheimer's amyloid precursor protein (APP), and tumour necrosis factor-α (TNF-α). The proteinases responsible for the release of these membrane proteins have been termed membrane protein secretases, sheddases or convertases. The secretases involved in the release of distinct membrane proteins have several properties in common. Many of the activities are zinc metalloproteinases, being inhibited by chelating agents and synthetic hydroxamic acid-based compounds such as batimastat [1]. In addition, the secretase activities are upregulated by phorbol esters, and cleave within the membrane proximal stalk region of the substrate protein [1].

ACE is a widely distributed ectoenzyme that occurs both as the membrane-bound form on the endothelial and epithelial surfaces of tissues and as a soluble form in plasma and other body fluids [2]. The secretase that releases ACE from the membrane is itself an integral membrane protein, localised to the plasma membrane [3–5]. Recently, we have shown that the secretase has an absolute requirement for its substrate (ACE) to be anchored in a membrane for cleavage to occur [4], a phenomenon that appears to apply to several other secretases including those responsible for the cleavage of APP [6,7], Kit ligand [8] and colony-stimulating factor [9]. ACE secretase is inhibited by several hydroxamic acid-based zinc metalloproteinase inhibitors including batimastat, marimastat and BB2116, with I_{50} values in the low micromolar range [4,10].

APP is cleaved by several proteinases termed α-, β- and γ-secretases [1,11,12]. Cleavage of APP at the N-terminus of the β-amyloid peptide by β-secretase and at the C-terminus by one or more γ-secretases constitutes the amyloidogenic pathway for processing APP. In the non-amyloidogenic pathway, α-secretase cleaves APP within the β-amyloid peptide, thereby preventing deposition of the intact amyloid peptide which is a primary constituent of the senile plaques in brains from Alzheimer's patients. Recently we have shown that α-secretase is inhibited by a range of hydroxamic acid-based compounds with a very similar structure activity relationship to that observed with ACE secretase [10]. These two secretases have several other properties in common. Both enzymes are stimulated by phorbol esters, cleave their respective substrates between a basic and a hydrophobic residue, and are integral membrane proteins with a distinctive detergent solubilisation profile [4–6,13]. Thus, ACE secretase and α-secretase appear to be the same, or closely related, zinc metalloproteinases.

TNF-α is cleaved from its membrane-bound precursor by TNF-α convertase [14]. The physiological importance of this inflammatory cytokine has led to intense interest in the mechanism of its generation, and the potential therapeutic benefit of inhibitors of this process [15]. TNF-α convertase (ADAM 17) has been recently isolated, cloned and sequenced [16,17]. The cDNA encodes a protein of 824 amino acids, consisting of a multidomain extracellular part, a transmembrane helix and an intracellular C-terminal tail. The extracellular part comprises an N-terminal pro-domain, a 259-residue catalytic domain containing the extended HEXXH zinc binding motif, and a Cys-rich domain that is composed of a disintegrin-like, an epidermal growth factor-like and a crambin-like domain. This domain structure is characteristic of the adamalysin or ADAMs (a disintegrin and metalloproteinase) family of zinc metalloproteinases [18,19]. The similarity in properties between TNF-α convertase and other secretases has led to speculation that TNF-α convertase may be involved in shedding other cell-surface proteins, in addition to TNF-α. In the present study we have investigated whether TNF-α convertase is able to cleave ACE in two cell-free systems. TNF-α convertase did not significantly cleave ACE, and the different inhibitor profile with three hydroxamic acid-based compounds, indicate that TNF-α convertase is distinct from ACE secretase and the amyloid precursor protein α-secretase.

*Corresponding author. Fax: (44) (113) 233 3167.
E-mail: n.m.hooper@leeds.ac.uk

Abbreviations: ACE, angiotensin converting enzyme; APP, amyloid precursor protein; TNF-α, tumour necrosis factor-α

PII: S0014-5793(98)00726-1

Table 1
ACE reconstituted into lipid vesicles is not significantly cleaved by TNF-α convertase

Enzyme	ACE cleaved (ng of ACE cleaved/min/μg enzyme)
Recombinant extracellular domain TNF-α convertase	0.17
Natural full-length TNF-α convertase	0.09
Trypsin	15.88

Affinity purified ACE (5 μg) was reconstituted into lipid vesicles and then incubated alone or in the presence of recombinant extracellular domain TNF-α convertase (0.5 μg), natural full length TNF-α convertase (15.4 ng) or trypsin (1 μg) for 2 h at 37°C in 10 mM Tris-HCl, pH 7.8. After the incubation the samples were subjected to phase separation in Triton X-114 and the resulting aqueous and detergent-rich phases assayed for ACE activity. Results are representative of two separate experiments each performed in duplicate.

2. Materials and methods

2.1. Materials

Batimastat (BB94) and compounds 1 and 4 were synthesised at SmithKline Beecham Pharmaceuticals, Harlow, UK. Compound 1 differs from batimastat only by the absence of the thienothiomethyl substituent adjacent to the hydroxamic acid moiety, while compound 4 differs from compound 1 by the presence of a tertiary amine at its C-terminus (for structures see [4]). Natural full-length TNF-α convertase and recombinant extracellular domain TNF-α convertase were provided by Dr R. Black (Immunex Corporation, Seattle, USA).

2.2. Reconstituted secretase assay

The amphipathic, full-length form of ACE was purified from porcine kidney cortex following solubilisation with Triton X-100 in the presence of 10 mM EDTA by affinity chromatography on lisinopril-2.8 nm-Sepharose [20]. Amphipathic, full-length ACE was reconstituted into dimyristoyl phosphatidylcholine vesicles as described previously [20]. After incubation of the lipid vesicles containing the amphipathic form of ACE (5 μg of protein) with either TNF-α convertase or trypsin at 37°C in 10 mM Tris-HCl, pH 7.8, the samples were subjected to temperature-induced phase separation in pre-condensed Triton X-114 [21]. ACE in the resulting detergent-rich and aqueous phases was quantitated by measurement of enzyme activity with BzGly-His-Leu as substrate. ACE secretase activity is quantitated as the release of ACE as determined by the amount of ACE in the final aqueous phase as a percentage of the total amount of ACE in both the aqueous and detergent-rich phases.

2.3. Angiotensin converting enzyme assay

ACE enzymic activity was determined with BzGly-His-Leu (5 mM) as substrate in 0.1 M Tris-HCl, 0.3 M NaCl, 10 μM $ZnCl_2$, pH 8.3. Reactions were terminated by heating at 100°C for 4 min, and the substrate and reaction products resolved and quantified by reverse-phase HPLC as described previously [22].

2.4. TNF-α convertase assay

TNF-α convertase was incubated with 833 μM TNF-α7 peptide (Ac-SPLAQAVRSSSR-NH_2; provided by Dr R. Black) for 30 min at 37°C in 10 mM Tris-HCl, pH 7.8. Following the incubation the reaction was stopped by heating at 100°C and the substrate and reaction products resolved and quantitated by reverse phase HPLC (Waters Millenium system) with a C18 column using a linear gradient of 0–50% MeCN in 0.1% trifluoroacetic acid with detection at 214 nm.

3. Results and discussion

The ability of TNF-α convertase to cleave ACE was assessed using the reconstituted assay system in which affinity purified ACE is incorporated into artificial lipid vesicles [4]. Lipid vesicles containing ACE were incubated with either recombinant extracellular domain TNF-α convertase or natural full-length TNF-α convertase (Table 1). The cleavage of ACE was examined by monitoring the distribution of the enzyme between the aqueous and detergent-rich phases following phase separation in Triton X-114. Negligible cleavage of ACE from the lipid vesicles by either form of TNF-α convertase was observed. In comparison trypsin readily cleaved ACE from the lipid vesicles (Table 1). For comparison, a detergent-solubilised porcine kidney microsomal membrane fraction, in which ACE secretase is present but not purified, cleaved ACE in the reconstituted assay with a specific activity of 0.03 ng ACE cleaved/min/μg protein [4].

In case there was the requirement for a factor present in biological membranes that was absent from the artificial lipid vesicles, the ability of recombinant extracellular domain TNF-α convertase to cleave and release ACE from porcine kidney microvillar membranes was examined. Incubation of the microvillar membranes at 37°C led to an increase in ACE activity in the aqueous phase due to the presence of endogenous secretase activity in the membranes (Table 2) as previously documented [3]. Addition of recombinant extracellular domain TNF-α convertase did not lead to an increase in the release of ACE from the microvillar membranes above that due to the endogenous secretase activity (Table 2). Batimastat, which inhibits both the endogenous secretase [4] and TNF-α convertase [17], completely inhibited the release of ACE from the microvillar membranes. The addition of trypsin to the microvillar membranes led to a significant increase in the release of ACE above that due to the endogenous secretase, indicating that the availability of substrate was not a limiting factor for the lack of increased cleavage in the presence of TNF-α convertase. Thus TNF-α convertase was unable to cleave and release ACE from a biological membrane system.

Previously we have described a structure/activity relationship for a series of hydroxamic acid-based, active site-directed zinc metalloproteinase inhibitors towards both ACE secretase [4] and APP α-secretase [10]. Using as substrate a synthetic peptide based on the cleavage site in pro-TNF [15], the inhibitory effect of three hydroxamic acid-based compounds on the activity of TNF-α convertase was examined (Table 3).

Table 2
TNF-α convertase does not release ACE from kidney microvillar membranes

Enzyme	ACE activity in aqueous phase (% of total)
No addition	34.9
TNF-α convertase	34.8
TNF-α convertase+batimastat	0.0
Trypsin	61.5

Porcine kidney microvillar membranes (76.2 μg of protein) prepared as described previously [24] were incubated alone or in the presence of recombinant extracellular domain TNF-α convertase (0.5 μg) or trypsin (7 μg) for 4 h at 37°C in 10 mM Tris-HCl, pH 7.8. Batimastat (100 μM) was included where indicated. Following the incubation, samples were subjected to phase separation in Triton X-114 and the resulting aqueous and detergent-rich phases assayed for ACE activity. The results are representative of two separate experiments each performed in duplicate.

Table 3
The effect of inhibitors on TNF-α convertase is different to that on ACE secretase and APP α-secretase

Inhibitor	TNF-α convertase I_{50} (μM)	ACE secretase I_{50} (μM)	α-secretase I_{50} (μM)
Batimastat	0.019 ± 0.002	1.6[b]	3.3[b]
Compound 1	0.037	38.3[a]	17.9[b]
Compound 4	0.18 ± 0.08	> 100[a]	> 20[b]

Natural full length TNF-α convertase (7.7 ng) was incubated in the presence of varying concentrations of the indicated inhibitors with 833 μM TNF-α7 peptide as described in Section 2. I_{50} values were determined from at least two independently determined inhibition curves. [a]Data from [4]. [b]Data from [10].

Although batimastat, compound 1 and compound 4 have very similar effects on ACE secretase and α-secretase, marked differences were observed in the inhibition of TNF-α convertase. All of the hydroxamate compounds examined were significantly (100–500-fold) more potent against TNF-α convertase than against the other two secretases, and at a concentration (100 μM) at which compound 4 failed to inhibit ACE secretase, complete inhibition of TNF-α convertase was observed. Compound 4 differs from compound 1 only by the presence of a tertiary amine at its C-terminus (for structures see [4]); a difference that makes compound 4 essentially inactive towards ACE secretase and α-secretase. The recently determined crystal structure of the catalytic domain of TNF-α convertase with a hydroxamate compound bound at the active site [23], revealed that the C-terminus of the inhibitor extends away from the active site cleft and adopts different conformations. It is possible that the active site clefts in ACE secretase and α-secretase make closer contact with the C-terminus of the inhibitor such that an amide H (as in batimastat and compound 1) is critical for efficient binding, whereas replacement of this group with a bulkier methyl group (as in compound 4) disrupts the interaction.

4. Conclusion

To date the secretases that cleave ACE and APP have not been identified. From the present study it would appear, using two cell-free systems in which the substrate is membrane-bound, that ACE is not significantly cleaved by TNF-α convertase. The difference in the effect of hydroxamic acid-based inhibitors towards TNF-α convertase on the one hand and ACE secretase and α-secretase on the other, further indicates that TNF-α convertase is distinct from, but possibly related to, these other two secretases. Whether ACE secretase and α-secretase are other members of the adamalysin family of zinc metalloproteinases awaits their isolation and sequencing.

Acknowledgements: We thank the Medical Research Council of Great Britain for financial support of this work. S.P. was in receipt of an Emma and Leslie Reid studentship from the University of Leeds. We thank Dr R. Black (Immunex Corporation, Seattle, USA) for the gifts of TNF-α convertase and TNF-α7 peptide.

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