A mosquito (*Anopheles stephensi*) angiotensin I-converting enzyme (ACE) is induced by a blood meal and accumulates in the developing ovary

U. Ekbote, D. Coates, R.E. Isaac*

School of Biology, University of Leeds, Leeds LS2 9JT, UK

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Abstract Angiotensin I-converting enzyme (ACE) has a key role in regulating levels of several circulating peptides in mammals and has a vital role in male fertility. ACE has recently been found in insects, where its role is unclear. A mutant allele of the ACE gene (Ance) of *Drosophila melanogaster* is embryonic lethal, indicating an important role for the enzyme in development. We now report the presence of ACE in female *Anopheles stephensi* mosquitoes and that the enzyme is induced by a blood-meal. ACE accumulates in developing ovaries and passes into the mosquito eggs, where it may play a role in the metabolism of peptides during embryogenesis. The ovarian ACE has an M_r of 70 kDa and is inhibited by captopril and lisinopril with IC_{50} values of 0.1 μM and 0.6 μM, respectively.

Key words: Angiotensin I-converting enzyme; Mosquito; Peptide metabolism; *Anopheles stephensi*

1. Introduction

Angiotensin I-converting enzyme (ACE, EC 3.4.15.1) is a dipeptidyl carboxypeptidase with a key role in the metabolism of bioactive peptides in the circulation and in tissues of mammals [1]. ACE, as part of the renin-angiotensin system, converts angiotensin I to the vasoconstrictor angiotensin II by removing the C-terminal dipeptide His-Leu and also contributes to the metabolic inactivation of the vasodilator peptide, bradykinin, by cleaving Arg-Phe from the C-terminus [2,3]. Mammalian ACE is also involved in the hydrolysis and inactivation of the haemoregulatory peptide, *N*-acetyl-SDKP [4]. Although only angiotensin I, bradykinin and *N*-acetyl-SDKP are confirmed in vivo substrates for mammalian ACE, in vitro studies demonstrate that ACE has a broad substrate specificity, even hydrolysing peptides with an amidated C-terminus, e.g. substance P, LH-RH, CCK, which can classify ACE as an endopeptidase[5]. Mammalian ACE exists as two isoforms, somatic ACE (sACE, M_r 150 000–180 000) and a testicular or germinal ACE (gACE, M_r, 100 000–110 000), uniquely expressed in developing spermatids [6]. Germinal ACE has an important, but unknown, role in reproduction since mice that do not express this isoform are infertile [7,8].

Recent studies show that ACE is an evolutionary ancient enzyme having been found in a number of invertebrates, including several species of insects [9]. The first insect ACE to be purified was from the housefly *Musca domestica* and biochemical studies showed that this enzyme has several enzymatic properties (substrate specificity, susceptibility to inhibitors of mammalian ACE, and activation by Cl^-) in common with mammalian ACE [10,11]. Similar enzymes have since been purified from two other dipteran insects, *Drosophila melanogaster* and the haematophagous buffalo fly, *Haematobia irritans* [12,13]. ACE from all three insects have a M_r of around 70 kDa and it is evident from cDNA sequences that both *D. melanogaster* and *H. irritans* ACE differ from the major form of the mammalian enzyme by the lack of a membrane anchor [12,13].

The strong similarity of the enzymatic properties of insect and mammalian ACEs suggest that the insect enzyme might also have an important role in the processing and inactivation of biologically active peptides. However, natural peptide substrates for insect ACE have not been identified and there is currently no evidence for the occurrence of insect peptides with similar structures to the known in vivo substrates of mammalian ACE (i.e. angiotensin I, bradykinin and AcSDKP). A strong mutant allele of the *D. melanogaster* ACE gene (known as Ance) is embryonic lethal indicating an important role in early development[14]. Flies carrying a weaker mutant Ance allele are viable, but adult males are sterile (A.D. Shirras, University of Lancaster, UK, personal communication). Thus, although the precise enzymatic role for insect ACE is not known, the enzyme appears to be vital for development and reproduction and may be considered a target for novel insect control. The expression of Ance in *D. melanogaster* is highly regulated during development with control exerted by zerknullt and decapentaplegic during embryogenesis [14] and possibly ecdysteroids during the pupal/adult moult [15]. In *H. irritans*, testes maturation is dependent upon a blood meal, which also induces the expression of ACE in the male reproductive tract [13].

In studies of the tissue distribution of ACE in various insect species, we have found ACE associated with both the male and female reproductive tracts. This paper reports for the first time the presence of ACE in the adult female mosquito *Anopheles stephensi*, a vector of human malaria, its induction by a blood meal and its accumulation in the developing ovary.

2. Materials and methods

2.1. Chemicals

All chemicals and immunological reagents were purchased from Sigma Chemical Co., Poole, Dorset, UK. The ECL^+ Western blotting detection system and the PVDF membrane was from Amersham International, Buckinghamshire, UK.

2.2. Insects

The mosquitoes were maintained at a constant temperature of 26.1–27°C at 82.5% relative humidity. The newly emerged adult male and female mosquitoes were fed on sterile 5% glucose/0.5% *p*-aminobenzoic acid solution and kept separate in a small netted cage (21 cm^3).

*Corresponding author. Fax: (44) (113) 2332835.
E-mail: r.e.isaac@leeds.ac.uk

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On the fourth day after emergence female mosquitoes were fed on mouse blood for 20 min and kept in the netted cages. Tissues were dissected in Ephrussi and Beadle Ringer's solution [16].

2.3. Assay of ACE activity in adult female Anopheles tissues

Whole insects and dissected tissues were homogenised in 50 mM Tris-HCl, pH 7.0, 1% (w/v) Triton X-100 and the resulting homogenates were then centrifuged at 13 000×*g* for 7 min. ACE activity in the supernatants was assayed by measuring the release of hippuric acid from the ACE substrate Hip-His-Leu as described previously [10]. Protein was measured by the bicinchoninic acid (BCA) method using a commercial kit (Sigma Chemical Co.) and bovine serum albumin as the protein standard.

2.4. PAGE and immunodetection of insect ACE by enhanced chemiluminescence (ECL)

PAGE was perfomed using a 10% SDS-polyacrylamide gel on a Mini Protean II Bio-Rad system run at 200 V for 45 min. Proteins were transferred to a PVDF membrane using a Bio-Rad transfer unit at 100 V for 1 h. The membrane was blocked with 5% dried milk in 0.05% Tween 20 in phosphate buffered saline (PBST) overnight at 4°C. Next day the membrane was washed 3 times in PBST at intervals of 6 min before incubation for 1 h with antiserum to *D. melanogaster* Ance, diluted 1:5000 in 5% dried milk/PBST. The membrane was washed a further 3 times in PBST before being incubated for 1 h with rabbit anti-rat IgG coupled to horseradish peroxidase diluted 1:5000 in PBST. After washing away the excess reagent with PBST, Ance antibodies were detected by chemiluminescence using an ECL$^+$ Western blotting detection system. Apart from the overnight incubation, all procedures were carried out at 20°C.

3. Results

3.1. Increase in ACE activity in adult female A. stephensi after a blood meal

HPLC was employed to detect the enzymatic release of hippuric acid on incubation of Hip-His-Leu with a homogenate of whole adult female *A. stephensi*. Hip-His-Leu is a substrate that mimics the C-terminus of angiotensin I and is routinely employed to assay for mammalian ACE [17] and has proven to be equally suitable for measuring insect ACE activity [10–12]. The mosquito ACE activity was linear with time and protein concentration and was completely blocked by captopril, a potent inhibitor of both mammalian and insect ACE.

The effect of a blood meal on ACE activity in whole adult females was investigated by comparing the level of enzyme activity in homogenates of 4 day old adults not fed with a blood meal, with 6 day old insects given a blood meal at day 4 of adult life. A 260% increase in the ACE activity in the adult female occurred following a blood meal (Table 1). This rise in ACE activity was also reflected in the near 2-fold increase in the specific activity of the enzyme from 0.163 ± 0.035 to 0.292 ± 0.045 nmol of Hip-His-Leu hydrolysed/min/μg of protein (± S.E.M., $n = 10$). The increase in the ACE activity was not the result of accumulation of mouse plasma ACE from the diet, since most of the blood in the mosquito gut was digested 48 h after the blood meal.

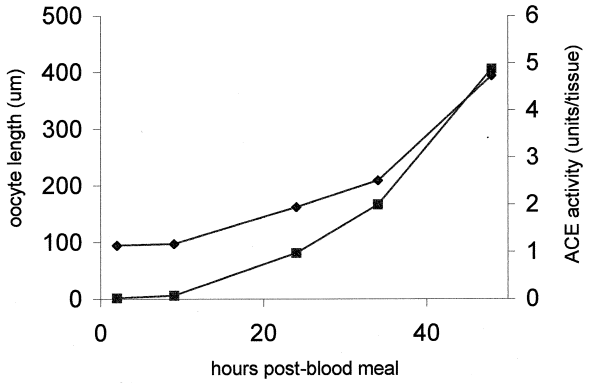

Fig. 1. ACE activity during oocyte maturation. Batches of 10 ovaries (1 batch for each time point) were dissected from blood-fed female *A. stephensi* and the lengths (◆) of the terminal oocytes were recorded before the ovary was homogenised in 50 mM Tris-HCl, pH 7.0, 1% (w/v) Triton X-100. After centrifugation at 13 000×*g* for 7 min, the soluble ovarian protein was assayed for ACE activity (see Section 2). A unit of activity is 1 nmol of Hip-His-Leu hydrolysed/min (■).

3.2. ACE activity in tissues of fed and unfed adult female A. stephensi

The levels of ACE activity in the ovary, gut and carcass (all remaining tissues) of adults 48 h after the blood meal were compared with the activity found in unfed adult females (Table 1). A large increase in activity from 0.02 to 7.33 nmol of Hip-His-Leu hydrolysed/min/tissue was recorded for the ovarian tissue, compared to a relatively small rise in the activity of the gut and the carcass. Thus the increase in enzyme activity observed previously in whole insects can be accounted for by the dramatic increase in the levels of ovarian ACE which was coincident with the development of the terminal oocytes and which reached a maximum level by 48 h, just prior to egg-laying (48–52 h) (Fig. 1). At the end of the first reproductive cycle, the ACE activity was entirely transferred from the ovaries to the newly laid eggs (Table 1).

A sample of a centrifuged homogenate of ovaries from 48 h post-fed insects was analysed by immuno-blotting of the ovarian proteins separated by SDS-PAGE, with an antibody raised to recombinant *Drosophila* Ance [15]. A protein with

Table 1
Levels of ACE activity in homogenates of tissues from adult female *A. stephensi* before and 48 h after a blood meal

Source of enzyme	ACE activity (units of activity/insect or tissue ± S.E.M.)	
	Before blood meal	After blood meal
Adult female	3.69 ± 0.47 ($n = 10$)	9.67 ± 1.59 ($n = 10$)
Ovary	0.02	7.33
Gut	0.01	0.04
Carcass	2.727 ± 0.131 ($n = 10$)	3.570 ± 0.494 ($n = 10$)
Ovaries after first reproductive cycle	0.02	–
Eggs (120) of the first reproductive cycle	6.2	–

ACE activity was assayed using Hip-His-Leu as the substrate (1 unit of activity = 1 nmol of Hip-His-Leu hydrolysed/min). The carcass included all tissues apart from the gut and the ovary. A homogenate of pooled ovaries, eggs and gut tissue was used to determine the levels of ACE activity in some tissues, because of the low levels of enzyme activity encountered.

an M_r of 70 000 was detected in the homogenate of mature ovaries, but not in a homogenate of immature ovaries taken from non-fed 4 day old adult females (Fig. 2). The ovarian ACE was inhibited by captopril and lisinopril, two potent inhibitors of mammalian ACE, with IC_{50} values of 0.1 and 0.6 μM, respectively (Fig. 3).

4. Discussion

Adult female *A. stephensi* possess a dipeptidyl carboxypeptidase that is inhibited by selective inhibitors of mammalian ACE and is induced by a blood meal. The induced enzyme accumulates in the developing ovary and is eventually found in newly laid eggs. Antiserum to *D. melanogaster* Ance, which does not cross-react with mammalian ACE (unpublished observations) nor with a second *D. melanogaster* ACE-related protein known as Acer [15], recognised a 70 kDa protein in mature ovaries from blood-fed insects, suggesting that the *A. stephensi* enzyme is a homologue of *D. melanogaster* Ance. The size of the mosquito protein is typical of insect ACEs [11,13,15,18], but quite different from the larger somatic (150–180 kDa) and germinal (90–110 kDa) forms of ACE found in mammals [17].

Essentially all of the elevated ACE found in adult female mosquitoes 48 h after a blood meal can be accounted for by the appearance of ACE in the ovary, but it cannot be assumed that the ovarian tissue is responsible for the synthesis of the new enzyme. It is possible that the *A. stephensi* ACE is synthesised extra-ovarially in a tissue such as the fatbody and that the enzyme is rapidly transferred to the oocytes. Such a mechanism has been described for a mosquito serine carboxypeptidase (VCP), which is synthesised in the fat body of the adult female *Aedes egypti* before being internalised by the oocytes and deposited in the yolk bodies of eggs [19].

The ingestion of blood by mosquitoes is the trigger that sets in train a series of endocrine events that control the reproductive cycle, the production of digestive enzymes, water and ion balance, and behaviour [20]. An early step in the signalling pathway is the release of a steroidogenic gonadotropin from medial neurosecretory cells in the brain, which in turn stimulates the synthesis of ecdysteroids by the ovarian follicle cells [21,22]. The release of the ovarian ecdysteroid into the haemolymph provides the signal for vitellogenin synthesis in the mosquito fatbody and may also control the expression of the VCP gene in *Aedes egypti*. The appearance of ovarian ACE in blood fed *A. stephensi* might be another response to the blood meal-induced release of ecdysteroids from the ovary.

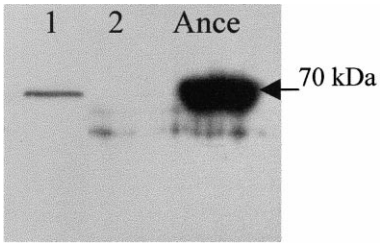

Fig. 2. Immunoblot of *A. stephensi* ovarian ACE with antibodies specific for *D. melanogaster* Ance. Equal amounts of protein (30 μg per lane) from homogenates of ovaries dissected from (1) blood-fed and (2) non-fed female insects were transferred together with 100 ng of Ance to a PVDF membrane after SDS-PAGE. The antiserum employed was raised against recombinant *D. melanogaster* Ance [15].

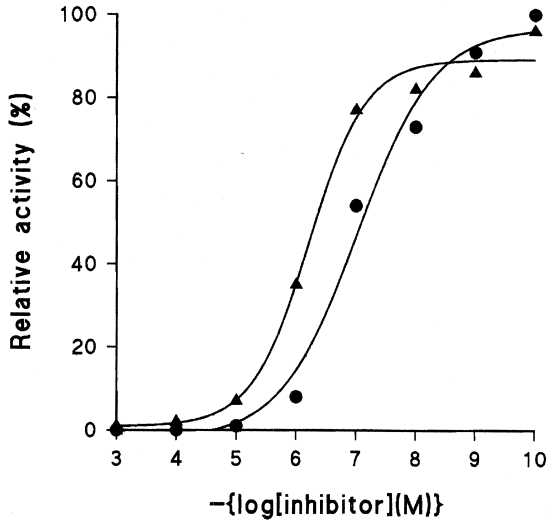

Fig. 3. Inhibition of mosquito ACE by captopril (▲) and lisinopril (●). ACE from a centrifuged homogenate of ovaries taken from adult females, 48 h after a blood meal, was assayed in the presence of different concentrations of inhibitor as described in Section 2. The enzyme was incubated with the inhibitor for 20 min before the addition of substrate. Data are expressed as a percentage of the uninhibited activity and are the mean of triplicate determinations.

The induced ovarian ACE of *A. stephensi* might have important roles in both the adult and in the developing embryo. Insect ACE, like the mammalian enzyme, removes C-terminal dipeptides from the C-terminus of oligopeptides, a process which can either confer or abolish biological activity depending on the peptide [11,13,23]. Recently it was shown that insect ACE is very efficient at trimming pairs of basic residues from the C-terminus of peptides resembling processing intermediates of the leucopyrokinin family of insect neuropeptides, suggesting a possible new role for ACE in pro-hormone processing [31]. The accumulation of ACE in the maturing ovary might be important for regulating the activity of peptides controlling oocyte development, whereas the transfer of maternal ACE to the egg suggests a possible role for the enzyme in generating regulatory peptides during embryogenesis. Female mosquitoes are known to possess a plethora of peptides that are likely to be involved in controlling the synthesis and release of digestive enzymes, the cyclic synthesis of vitellogenins, fluid and electrolyte homeostasis, and behaviour [21,24–30]. ACE might be responsible for regulating levels of some of these peptides during and after the blood meal. Many of the known mosquito peptides possess an amidated C-terminus, which protects the peptide from degradation by carboxypeptidases. In contrast, insect ACE can inactivate amidated peptides by removing C-terminal dipeptide amides [23]. However, a clearer understanding of the physiological role of the inducible ovarian ACE of *A. stephensi* will require the identification of the peptide substrates.

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