ACE and ACE2: insights from Drosophila and implications for COVID-19

Paul Herrera a,b, Ruben J. Cauchi a,b,*

a Centre for Molecular Medicine and Biobanking, Biomedical Sciences Building, University of Malta, Msida, Malta
b Department of Physiology and Biochemistry, Faculty of Medicine and Surgery, University of Malta, Msida, Malta

1. Introduction

Angiotensin-converting enzyme (ACE) and its homologue ACE2 are key regulators of the renin-angiotensin system and thereby cardiovascular function through their zinc-metallopeptidase activity on vasoactive peptides. ACE2 also serves as the receptor for the cellular entry of various coronaviruses including the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is responsible for the coronavirus disease 2019 (COVID-19). The unprecedented scale of the COVID-19 pandemic has spurred the use of mammalian models to investigate the SARS-ACE2 relationship and knowledge gained from such research has accelerated development of vaccines and therapeutics. Recent studies have just started to underscore the utility of the fruit fly Drosophila melanogaster as a model system to study virus-host interactions and pathogenicity. Notably, the remarkable existence of catalytically functional ACE and ACE2 orthologues in Drosophila, discovered more than two decades ago, provides a unique opportunity for further developing this model organism to better understand COVID-19 in addition to identifying coronavirus preventative and therapeutic interventions targeting ACE2. Here, we review the studies that revealed crucial insights on the biochemistry and physiology of Ace and Acer, two out of the six Drosophila ACE family members with the greatest homology to human ACE and ACE2. We highlight shared in vivo functions outside of the renin-angiotensin system, which is not conserved in flies. Importantly, we identify knowledge gaps that can be filled by further research and outline ways that can raise Drosophila to a powerful model system to combat SARS-CoV-2 and its threatening vaccine-evading variants.
COVID-19 recovering patients (Shu et al., 2021). It is interesting that the absolute majority (90%) of human proteins in the SARS-CoV-2 human interactome are conserved in Drosophila (van de Leemput and Han, 2021; Zhu et al., 2021) and the identification of chemicals that mitigate phenotypes linked to SARS-CoV-2 protein expression show the potential of flies as a powerful in vivo drug discovery platform (Yang et al., 2020; Zhu et al., 2021). However, most remarkable is the existence of catalytically functional ACE and ACE2 orthologues in Drosophila. Here we provide a timely review of classic and recent studies that reveal insights on their structure, biochemistry, substrate specificity, expression, regulation, tissue distribution and function. Importantly we ask whether additional research aimed at addressing outstanding questions is warranted to further develop Drosophila as an additional in vivo model for understanding COVID-19 and, crucially, in preparation for future coronavirus outbreaks.

2. Discovery of ACE and ACE2 orthologues in Drosophila

ACE and ACE2 can be regarded as evolutionarily ancient enzymes when one takes into consideration the existence of orthologues in several mammalian, insect and bacterial species (Lubbe et al., 2020). Whereas in humans the number of ACE genes appears to be limited to ACE and ACE2 (excluding non-expressed pseudogene ACE3), in Drosophila, there are six ACE-like genes (Table 1). Out of these, only Ance and Acer, the sole genes whose products are predicted to have zinc-metallopeptidase activity (Coates et al., 2000), have been very well characterised. All genes encode for proteins with one active site domain, a characteristic that renders them similar to mammalian ACE2 and the germinal or testicular form of ACE (gACE or tACE) (Figure 1). The latter is one of two forms derived from the mammalian ACE gene through use of alternative promoters. The other form is the larger somatic ACE (sACE) containing two active site domains, likely resulting from gene duplication (Hooper et al., 2020; Crackower et al., 2002; Jia et al., 2020; Krege et al., 1995), Drosophila Ance and Acer are not essential genes (Table 1). Homozygous Ance mutants generated through chemical mutagenesis were viable despite a reduction in survival to adulthood with sterility observed in adult male flies (Hurst et al., 2003). Acer (Angiotensin converting enzyme-related) was identified in 1996 through a cDNA clone from an embryonic library. During embryogenesis, expression of Acer was apparent in the developing heart (Taylor et al., 1996). In adults, Acer is strongly expressed within the fat body in both the head and abdomen (Carhan et al., 2011). The remaining four ACE-like genes including Ance-2 and Ance-3, which are located adjacent to the Ance gene, were identified in 2000 as a result of the sequencing of the complete D. melanogaster genome (Coates et al., 2000). Similar to mammalian ACE and ACE2 (Cole et al., 2000; Crackower et al., 2002; Jia et al., 2020; Krege et al., 1995), Drosophila Ance and Acer are not essential genes (Table 1). Homozygous Ance mutants generated through chemical mutagenesis were viable despite a reduction in survival to adulthood with sterility observed in adult male flies (Hurst et al., 2003). However, a recent study describing Ance deletion alleles showed that homozygous flies were viable, fully fertile and morphologically normal (Kim et al., 2017). Acer null homoygotes were reported to be adult viable and fertile, though adults experienced a disrupted circadian behaviour (Carhan et al., 2011).

3. Enzymatic properties and specificity

Expression of recombinant Ance and Acer in yeast and their eventual detection in the culture medium confirmed that both enzymes are secreted, as expected from the lack of a membrane anchor domain (Houard et al., 1998). Mimicking the cardinal activity of its mammalian orthologue ACE (Lubbe et al., 2020), Drosophila Ance is capable of converting Ang I to Ang II and hydrolysing the vasodilatory peptide, bradykinin (Cornell et al., 1995; Houard et al., 1998). Glycosylation is not required for its secretion and enzymatic activity; however, it improves

Table 1. Characteristics of the ACE family members in humans and Drosophila. The active site domain of both Ance and Acer share the typical zinc-binding HEXXH + E (where X is any amino acid) consensus sequence (only partially present in Ance-3) found in both ACE and ACE2 (underlined). Enzymatic activity refers to the prediction of zinc-metallopeptidase activity based on the presence or absence of the zinc-binding motif.

| Protein | Protein length (aa) | Predicted TM domain | Active site region | Essential for adult viability | Enzymatic activity |
|---------|---------------------|---------------------|--------------------|-----------------------------|-------------------|
| ACE     | 1306                | Yes                 | HHEMGHQY...HEAE (N-terminus) | No                          | Yes              |
| ACE2    | 805                 | Yes                 | HHEMGHQY...HEAV (C-terminus) | No                          | Yes              |
| Acer    | 630                 | No                  | HHELGHOY...HEAV     | No                          | Yes              |
| Ance    | 615                 | No                  | HHELGHOY...HEAV     | No                          | Yes              |
| Ance-2  | 611                 | No                  | FEAQSLQY...SDAIG    | NA                          | No               |
| Ance-3  | 844                 | Yes                 | HHEMAHQYF...IQAV    | NA                          | No               |
| Ance-4  | 609                 | No                  | HGTMAEQY...GAIA     | NA                          | No               |
| Ance-5  | 628                 | No                  | HSHMARVYYA...EFAV  | NA                          | No               |

Abbreviations: TM, transmembrane; NA, not available.
protein stability (Williams et al., 1996). Although, Acer does not cleave Ang I, even at high concentrations (Houard et al., 1998), it is able to cleave bradykinin, albeit with an efficiency lower than that observed for Ance and human ACE (Bingham et al., 2006). This property makes Acer somewhat similar to ACE2 which is itself unable to cleave bradykinin (Hooper et al., 2020; Lubbe et al., 2020). Acer and Ance can also act as endopeptidases, a property also observed for mammalian ACE (Lubbe et al., 2020). Hence, both Acer and Ance were shown to cleave the C-terminal dipeptide amides from [Leu5]enkephalinamide and synthetic substrate hippuryl-L-histidyl-L-leucine-NH₂ (HHL-NH₂) as well as the C-terminal dipeptides from [Leu5]enkephalin and HHL. However, Acer hydrolyses the latter six-fold less efficiently than Ance (Houard et al., 1998). Overall, Acer displays more restricted substrate specificity compared to Ance (Siviter et al., 2002a). Both Ance and Acer can be inhibited by ACE inhibitors captopril, trandolaprilat and enalaprilat with slight variations noted, most probably the result of active site differences (Cornell et al., 1995; Houard et al., 1998).

Although the two domains of human sACE share enzymatic abilities including the ability to hydrolyse bradykinin and Ang I, they possess distinct substrate specificities (Wei et al., 1991) and differential inhibitor preferences (Dive et al., 1999; Wei et al., 1991, 1992). Furthermore, they vary in the degree to which they are activated by NaCl, with the C-domain active site being more sensitive to changes in Cl⁻ ion concentration (Wei et al., 1991). Ance has a strong functional resemblance to the C-domain of sACE such as the hydrolysis of substrates at comparable rates and equivalent chloride concentration requirements (Lubbe et al., 2020; Williams et al., 1996). In contrast, Acer shares structural features with the N-domain of human sACE, confirmed by the ability of N-domain selective inhibitor RXP407 to potently inhibit Acer, but not Ance. This indicates that like the sACE N-domain, the active site of Acer has a more relaxed specificity compared to that of sACE C-domain and Ance (Coates et al., 2000). Unexpectedly, Acer was also shown to be weakly inhibited by RXPA380, a highly selective inhibitor of the sACE C-domain (Bingham et al., 2006).

Structural studies of Ance bound or unbound to its ligands have revealed various insights on its structure-function relationships and those of its human orthologues (Akif et al., 2010a, 2010b, 2011, 2012; Harrison and Acharya, 2015; Masuyer et al., 2014). However, no bound Cl⁻ ions were recognised in the crystal structure of Ance in contrast to that observed in the structures of human ACE and ACE2 (Guy et al., 2005; Kim et al., 2003; Natesh et al., 2003, 2004), indicating that Cl⁻ ion binding sites in Ance are different or absent. This would explain the relatively weaker effect of NaCl on Ance's enzymatic activity (Bingham et al., 2006). Similarly, a model of the structure of Acer predicts a lack of Cl⁻ ion binding sites but the strong activity towards enkephalinamide peptides by NaCl suggests that Cl⁻ ions bind to alternative sites. Marked differences in the electrostatic charge of the substrate channel are however observed between Ance and Acer. Structural variations including different amino acids at select positions explain differences in inhibitor selectivity and potency (Bingham et al., 2006). The negative charges lining the Ance substrate channel (in contrast to the positively charged active site in Acer) favour interactions with positively charged peptide substrates. This explains the efficient cleavage of electropositive peptides such as bradykinin by Ance (Bingham et al., 2006) and, importantly, the
binding to the positively-charged receptor-binding domain (RBD) of the SARS-CoV-1 spike (S) glycoprotein to ACE2 (Prabakaran et al., 2004). Spike proteins decorate the coronavirus envelope, hence, the eponymous crown-like (corona) structure.

4. In vivo functions

RAS substrates are not conserved in Drosophila which raises questions about the in vivo functions of the ACE and ACE2 orthologues in this organism (Figure 2). Nonetheless, phenotypes observed on disruption of Ance and Acer are strikingly similar to those observed in mouse models. Amongst other features, ACE mouse knockouts display male infertility (Hugaman et al., 1998; Krege et al., 1995) although this is due to loss of a RAS-independent function, considering that angiotensinogen knock-out mice are themselves fertile (Kim et al., 1995). Similarly, male Ance mutants are sterile with testes lacking individualised sperm, indicating a role for Ance in spermatid differentiation and individualisation (Hurst et al., 2003). Ance is thus present in high concentrations in testes, accumulating in vesicles in spermatocytes (Hurst et al., 2003), and in a similar manner, gACE is enriched in murine male germ cells (Sibony et al., 1994). Ance and its mammalian counterpart might have an as yet undefined but evolutionary-conserved role in the processing of peptides secreted by germ cells. A RAS-dependent function for mammalian sACE has also been proposed to occur in the prostate (Leung and Sernia, 2003; O’Mahony et al., 2005; O’Mahony et al., 2000), which is critical for the production of seminal fluid. The Drosophila equivalent of the prostate is the male accessory gland (AG), and Ance was shown to also be synthesised by this organ. Ance was found to be expressed in the secondary cells of the AG, specifically found enriched within the large vesicles of these cells (Rylett et al., 2007). ACE activity, determined by detecting HHL proteolysis, was lost from the AG during mating, consistent with the knockdown of Acer expression was found to exacerbate the contractility (Crackower et al., 2002). A role for ACE2 in cardiac function is remarkably conserved in Drosophila larvae (Houard et al., 1998). Activity in newly eclosed adults then declines to that observed in pre-pupal stages. The observed increase in ACE activity, as measured by analysing HHL hydrolysis, is due to the induction of Ance expression in cells of imaginal discs in wandering third instar larvae. This induction is absent in mutants that fail to produce the ecdysone peak during the wandering larval phase, which is required to trigger puparium formation. The synthesis of ACE activity in addition to Ance protein expression brought about by physiological levels of 20-hydroxyecdysone in a wing disc cell line confirms that Ance is an ecysteroid-responsive gene (Sliviter et al., 2002b). It has thus been postulated that, during metamorphosis, Ance is required for the processing of a developmental peptide hormone or may function in concert with other peptideas to recycle amino acids from larval proteins for use in the synthesis of adult proteins (Sliviter et al., 2002b). A recent study has dissected the mechanism through which expression of Ance is regulated in imaginal discs by revealing a requirement for Decapentaplegic (Dpp) signalling transcription factor Mad and GATA family transcription factor Pannier. This mechanism appears to be conserved in humans since ACE expression was found to be regulated by Mad and Pannier homologues SMAD2 and GATA4 (Kim et al., 2017).

ACE inhibitors and blockers are associated with a significant reduction in the incidence and progression of Alzheimer's disease (AD) (Davies et al., 2011; de Oliveira et al., 2014; Ho et al., 2017; O’Coimh et al., 2014; Ohrui et al., 2004; Qiu et al., 2013; Soto et al., 2013; Wharton et al., 2015; Yasar et al., 2013). Interestingly, administration of ACE inhibitor captopril and angiotensin receptor blocker losartan was found to suppress neurodegenerative phenotypes in AD fly models, although neither drug affected the production, accumulation or clearance of Aβ42. Notably, neurodegenerative changes such as brain cell death and memory deficits were completely rescued in a homozygous Acer null background, hence demonstrating that the beneficial effect of captopril results from its targeting of Acer (Lee et al., 2020). In corroboration, an earlier study identified Acer and Ance-5 as genetic modifiers of phenotypes
associated with *Presenilin and Amyloid Precursor Protein* genes, mutations in which lead to early-onset autosomal dominant AD (van de Hoef et al., 2009). Considering that Ance is also a target of captopril (Kim et al., 2003), it remains to be seen whether Ance can also modify AD phenotypes in fly models. Treatment of flies with ACE inhibitor lisinopril was found to increase lifespan and physical performance, though the latter was genotype-dependent. Genotypes in which lisinopril was found to enhance motoric abilities had a reduction in age-related protein aggregation in muscle. Reduced levels of Ance in muscles achieved through RNAi-mediated knockdown abolished the effects of lisinopril on lifespan. This implies that the ameliorative effects of lisinopril on lifespan are most probably due to its targeting of Ance in muscle (Gabrawy et al., 2019). Lisinopril was also found to alter mitochondrial respiration and reactive oxygen species (ROS) levels in an age- and genotype-specific manner. These changes can also contribute to the drug's positive impact on age-related impairments (Ederer et al., 2018). All these findings indicate that modulation of RAS-independent functions can have a modifier effect on ageing and age-related diseases with implications for treatment development. Importantly, they highlight the need for continued exploration of the non-canonical functions of ACE and ACE2 with *Drosophila* showing great promise in this pursuit considering that this model organism has an open circulatory system (Figure 2).

5. Outstanding questions

Despite extensive research on ACE and ACE2 *Drosophila* orthologues Ance and Acer, several questions still remain to be answered (Box 1). The substrates of the two enzymes catalyse in vivo remain undiscovered. However, advances in proteomics aided by the availability of null mutants can help shed light on this gap in knowledge. Although *Drosophila* ACE-like proteins other than Ance and Acer are not predicted to be catalytically active, further research is warranted to test whether this hypothesis stands in an *in vivo* setting. Specifically, the functions of Ance-2, Ance-3, Ance-4 and Ance-5 in *Drosophila* remain unexplored and the availability of easy-to-use gene editing techniques including the CRISPR/Cas9 system to generate fly knockouts and/or RNAi transgenes, is expected to first and foremost determine whether these are essential genes. Secondly, such tools will allow us to identify tissue-specific roles. Few studies have thus far applied RNAi to select tissues. In one study, findings were crucial to confirm that the cardiac function ascribed for ACE2 is conserved for its Acer orthologue in *Drosophila* (Liao et al., 2014). Studies in the same vein will help us to not only define the unknown function of Ance-2 to Ance-5 but also to redefine the *in vivo* role of Ance and Acer. It would also be interesting to determine whether the two-domain membrane-anchored Ance-2+Ance-3 fusion protein is expressed in flies, potentially by generating and characterising flies with transgenic expression of this hypothetical protein thought to have the greatest structural resemblance to ACE and ACE2. Site-directed mutagenesis can also be employed to activate those ACE proteins thought to be catalytically inactive.

In addition to discovering possible genetic interactions, combinatorial mutant analysis will also help determine if mutant combinations are compatible with life, therefore, elucidating whether some ACE family members are redundant. This approach can also determine whether specific mutant-associated defects can be rescued in a combined mutant background similar to what was observed in *Ace/Ace2* double mutant mice (Crackower et al., 2002). Thus far, there is indication that double mutants for *Ance and Acer* are developmentally normal and fertile (Kim et al., 2017). Extensive genetic modifier screens can also reveal relevant pathways in mutants. The use of fluorescent tags, specific antibodies and immunofluorescence to identify tissue-specific expression patterns has already been quite informative in the study of Ance function (Carhan et al., 2011; Hunt et al., 2003; Kim et al., 2017; Rylett et al., 2007), and studies that apply these methods to the other ACE family members in *Drosophila* are expected to yield valuable results. This would be especially relevant for Ance-3 which is the only *Drosophila* ACE protein that is predicted to be membrane-bound in a similar manner to ACE and ACE2.

Given the societal impact of COVID-19, investigations that discover an overlap between the clinical features observed in COVID-19 patients, including those with long COVID, and phenotypes exhibited by flies with disruption of ACE family members will help elucidate the pathophysiology of this disease and its ramifications. It would also be interesting to determine whether ACE2 orthologues in *Drosophila*, like the murine ACE2 counterpart (Qiu et al., 2020; Wan et al., 2020), have a similar low binding affinity to the SARS-CoV-2 spike protein. Should this be the case, adopting the strategy utilised in mouse models (Jia et al., 2020; Munoz-Fontera et al., 2020), development of humanised ACE and ACE2 fly models through the transgenic expression of human ACE or ACE2 in either a wild-type or mutant background will be of great value to the research community. Such models will allow us to first determine whether the function of any or all ACE family members can be replaced by either or both human orthologues. Importantly, a transgenic ACE2 fly model can be a crucial tool to further understand ACE2 biology, investigate its role in the pathogenesis of coronavirus diseases including COVID-19 or its variations, and identify novel ACE2 inhibitors. The economical and expeditious platform afforded by *Drosophila* will be key for the discovery of treatments that complement those in the pipeline, making us more prepared for future coronavirus epidemics.

6. Conclusions

More than two decades have passed since the discovery of the first ACE family members in *Drosophila* and various studies have since shaped our understanding of their *in vivo* roles and the functional overlaps with human orthologues ACE and ACE2. The unprecedented scale of the COVID-19 pandemic, which has resulted in more than 200 million cases and 5.3 million deaths to date worldwide (Johns Hopkins University Coronavirus Resource Center: https://coronavirus.jhu.edu), has spurred us to re-evaluate the research done on ACE orthologues in *Drosophila*, aimed at determining whether the advantages offered by the fruit fly as a systems model can be exploited in our ongoing struggle against SARS-CoV-2 and its threatening vaccine-evading variants. We conclude that *Drosophila* provides an enticing opportunity to further our understanding of ACE as well as ACE2 in humans and the link of the latter to COVID-19. Identifying preventative and therapeutic interventions targeting ACE2 is feasible with the generation of humanised models being essential for this endeavour. We believe that research on ACE/ACE2 in flies provides an added value and, importantly allows us to be better prepared for the next coronavirus pandemic.
Declarations

Author contribution statement

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The authors declare no conflict of interest.

Additional information

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