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Original Article

Isolation of the monooxygenase complex from *Rhipicephalus (Boophilus) microplus* – clues to understanding acaricide resistance

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ABSTRACT

The monooxygenase complex is composed of three key proteins, a cytochrome P450 (CYP), the cytochrome P450 oxidoreductase (CPR) and cytochrome b_5 and plays a key role in the metabolism and detoxification of xenobiotic substances, including pesticides. In addition, overexpression of these components has been linked to pesticide resistance in several important vectors of disease. Despite this, the monooxygenase complex has not been isolated from the Southern cattle tick *Rhipicephalus* (*Boophilus*) *microplus*, a major disease vector in livestock.

Using bioinformatics 115 transcriptomic sequences were analyzed to identify putative pesticide metabolizing CYPs. RACE-PCR was used to amplify the full length sequence of one CYP; CYP3006G8 which displays a high degree of homology to members of the CYP6 and 9 subfamilies, known to metabolize pyrethroids. mRNA expression levels of CYP3006G8 were investigated in 11 strains of *R. microplus* with differing resistance profiles by qPCR, the results of which indicated a correlation with pyrethroid metabolic resistance. In addition to this gene, the sequences for CPR and cytochrome b_5 were also identified and subsequently isolated from *R. microplus* using PCR.

CYP3006G8 is only the third CYP gene isolated from *R. microplus* and the first to putatively metabolize pesticides. The initial results of expression analysis suggest that CYP3006G8 metabolizes pyrethroids but further biochemical characterization is required to confirm this. Differences in the kinetic parameters of human and mosquito CPR in terms of NADPH binding have been demonstrated and could potentially be used to design species specific pesticides. Similar differences in the tick CPR would confirm that this is a characteristic of heamatophagous arthropods.

1. Introduction

The detoxification of xenobiotics is an essential function for life and is carried out by three specific enzyme families: Glutathione S transferases, Esterases (or Carboxylesterases) and the Cytochrome P450s (CYPs). Cytochrome P450s are an ancient protein family found in all forms of life (Scott and Wen, 2001; Sztal et al., 2012) and are known to be the major phase I metabolizing enzymes. In humans, CYPs metabolize approximately 65% of currently used clinical drugs (Zhou et al., 2005), emphasising the importance of this group of enzymes in xenobiotic metabolism. CYPs are characterized by the presence of a highly conserved heme-binding domain located towards the C terminus of the protein. This signature motif, with the sequence FxxGxxxCxG is found in all CYPs (Syed and Mashele, 2014; Werck-Reichhart and Feyereisen, 2000) and plays a vital role in the electron transport mechanism used by these enzymes to, in many cases, incorporate molecular oxygen into xenobiotics thus rendering them more water soluble and more readily excreted from the cell (Mamidala et al., 2011).

Xenobiotic metabolism is an important topic of investigation amongst arthropods due to this group of organisms containing numerous vectors of medical/veterinary diseases and a host of agricultural pests that are developing resistance to commonly used chemical control agents (Brogdon and McAllister, 1998). Amongst the arthropods, there has been a huge amount of research into species such as *Musca domestica* (house fly) and *Drosophila melanogaster* (fruit fly) due to their use as model organisms and of course various mosquito species due to the medical and economic importance (Drali et al., 2012; Kawada et al., 2011; Kushwah et al., 2015; Wondji et al., 2011).

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Pesticide resistance is not a novel problem but one that is ever increasing due to its association with disease transmission to both animals and plants. Pesticide resistance has been described by the World Health Organisation (WHO) as "the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within the limits of tolerance of the subject" Abbas et al. (2014). It is this ability to survive despite the application of pesticides that is of interest as it provides a mechanism to continue the transmission of diseases to both plants and animals. One of the most studied of the arthropods with a view of disease transmission are the mosquito species and research has shown that CYPs play a key role in the development of pesticide resistance (Corbel et al., 2007; Gong et al., 2005; Hemingway et al., 2004; Marcombe et al., 2009). Cytochrome P450s are split into clades depending on their function and this allows key CYPs to be more easily identified. Four main clades (mitochondrial clade, CYP2, CYP3 and CYP4 clade) exist and in terms of pesticide metabolism the clade that is of interest is the CYP3 clade, a clade which includes the important mammalian CYP3A4 and insect homologues of this enzyme, namely the CYP6 and CYP9 families e.g. CYP6Z1 (Chiu et al., 2008) and CYP6P3 (Muller et al., 2008).

Despite the importance of ticks, as they are second to mosquitoes as vectors of human disease and the most important vector of disease to animals (Hill and Wikel, 2005), with the ability to transmit a variety of pathogens and transmitting the widest array of disease causing organisms out of all the hematophagous arthropods (Bissinger and Roe, 2010), little research has been carried out into the cytochrome P450 mediated metabolism and their subsequent associated resistance in this group. The vast majority of pesticide resistance research in these arthropods has focused on the role of GSTs (Kwon et al., 2010) and carboxylesterases (Baxter and Barker, 1998; Cossio-Bayugar et al., 2002), with the involvement of CYPs only being inferred by synergist studies (Li et al., 2004; Rodriguez-Vivas et al., 2006). Furthermore, despite the ever increasing problem of pesticide resistance little of this research has involved deciphering the mechanisms underlying the development of resistance phenotypes. The identification of CYPs, and indeed other drug metabolizing enzymes, in acari is hindered by the lack of available annotated genome data. Some data is available on public databases such as NCBI for *Ixodes scapularis* and the *I. scapularis* genome has been published (Van Zee et al., 2007) however for species such as *R. microplus*, the data available is even more sparse (Guerrero et al., 2006). It is therefore important to develop these resources such that a bioinformatics approach can be initiated to identify members of the key CYP families and where possible, identify putative homologues of the key insect CYPs that have been isolated and characterized to ascertain what they metabolize.

In order to be fully functional cytochrome P450s require an oxidoreductase (CPR in this case) and additionally in some cases cytochrome b_5. CPR is important in transferring electrons from NADPH through to the CYP allowing the incorporation of one molecule of oxygen, hence a monooxygenase reaction, to various substrates (Guengerich, 2007). CPR is a highly conserved diflavin protein composed of two flavin cofactors, flavin mononucleotide (FMN) and flavin adenenine dinucleotide (FAD), that transfer the electrons one at a time to a CYP (Iyanagi et al., 2012; Lian et al., 2011). Due to the nature of CYP research among mammals and insects, the CPR gene has been isolated from an array of species and been characterized in many of these (Chen and Zhang, 2014; Liu et al., 2014; Zhu et al., 2012) however, CPR has not been isolated or characterized from a tick species to date, despite the importance of this protein to the functioning of the monoxygenase complex and subsequent detoxification of xenobiotics. Differences have been identified between human CPR and *An. gambiae* CPR which is potentially paving the way for a new target and method of control (Lian et al., 2011), but further emphasises the importance of characterising CPR isoforms from other arthropods.

Cytochrome b_5 is a small but highly conserved protein with a variety of roles in different reactions such as lipid and sterol biosynthesis (McLaughlin et al., 2010; Porter, 2002; Zhang et al., 2005). The role cytochrome b_5 plays within the monooxygenase complex and CYP mediated reactions has been described as controversial for many years (Porter, 2002) as cytochrome b_5 has been shown to have a stimulatory/modifying effect on CYP reactions, be it obligatory for some reactions and in other cases have an inhibitory effect (Im and Waskell, 2011; Porter, 2002; Schenkman and Jansson, 2003). Despite this extensive amount of research into the role of cytochrome b_5 in CYP-mediated drug metabolism in mammals, much less equivalent research exists in arthropods considering the fact cytochrome b_5 may have a role in pesticide metabolism. Stevenson et al. (2012) found that by co-expressing numerous CYPs such as CYP9J2 from *Ae. aegypti* with cytochrome b_5 from *An. gambiae* the catalytic activity of the CYP was enhanced. Conversely, Chandor-Proust (2013) found that co-expression of *Ae. aegypti* cytochrome b_5 with *Ae. aegypti* CYP6Z8 did not cause a significant effect on the activity of the CYP (Chandor-Proust et al., 2013). This emphasises the need to co-express cytochrome b_5 when investigating CYP activity. Thus it is reasonable to assume that further investigation into the role of cytochrome b_5 in humans and some mosquito species will identify potential differences between species that similar to CPR, may additionally be exploited in the development of arthropod control measures.

In this paper we provide an update of our recent work towards filling the gap in the current knowledge of cytochrome P450s involved in pesticide metabolism in acari describing the isolation of the P450 monooxygenase complex from the Southern cattle tick, *R. microplus.* Isolation of these individual components will allow detailed biochemical characterization and the ascertainment of their role in pesticide metabolism/resistance.

2. Materials & methods

2.1. Bioinformatic analysis

115 putative cytochrome P450 sequences from *Rhipicephalus (Boophilus) microplus* provided by Dr Felix Guerrero and colleagues (Guerrero et al., 2006) were initially analyzed using the National Center for Biotechnology Information (NCBI) public database (http://www.ncbi.nlm.nih.gov/) to identify those having nucleotide sequence similarity to CYP3, CYP6 and/or CYP9. Of the 115 initial sequences 5 were identified as having the highest 'query coverage' and 'max ident' to known pesticide metabolizing CYPs from other species. Within these 115 sequences *R. microplus* homologues of cytochrome P450 oxidoreductase (CPR) and cytochrome b_5 (cytb$_5$) genes were also identified.

2.2. Tick samples

Tick larval samples from the species *R. microplus*, were provided from USDA, Texas (courtesy of Dr Felix Guerrero) preserved in RNA*later*-Ice® (Life Technologies, New York, USA), and stored at -80 °C to maintain RNA stability and integrity.

2.3. RNA Extraction and cDNA preparation

RNA was extracted from all 11 strains of *R. microplus* shown in Table 1 (the resistance profile of each strain is also included). The ticks were snap frozen using liquid nitrogen and RNA was extracted using TRIzol® reagent followed by a PureLink RNA Mini