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A new lipid carrier protein in the cattle tick *Rhipicephalus microplus*

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ABSTRACT

Tick infestation in cattle reflects the main cause of economic loss to cattle producers. This is due to several reasons but mainly to their ability to feed on blood and generate a huge amount of eggs. Lipid transport in arthropods is achieved by highly specialized hemolymphatic lipoproteins, which resemble those described in vertebrate blood. Such lipoproteins continuously deliver lipids through the blood to growing eggs. The injection of radioactive [^{3}H] palmitic acid into tick hemocoel showed that the gut, ovary, fat body and Gene's organ were the main organs of incorporation of this labeled fatty acid. The rate of [^{3}H] palmitic acid incorporation by the organs was high up to 30 min after injection. The [^{3}H] palmitic acid incorporated by these organs was later found in phospholipids and neutral lipids. Here, we describe the purification and characterization of a key player of lipid dynamics in tick hemolymph. The *Rhipicephalus microplus* lipid-apolipoprotein complex (RmLCP) is a new high-density lipoprotein (1.18 g/mL), which accounts for over 90% of [^{3}H] palmitic acid present in the hemolymph. It has a native molecular weight of 420 kDa and is composed of one subunit of 122 kDa. Protein identification analysis of RmLPC subunit showed two better hits: vitellogenin 2 (23% protein coverage) and vitellogenin 5 (29% protein coverage), respectively and similarities with hemolymphatic apolipoproteins of arachnids such as the tick *Ixodes scapularis* (80%), the mite *Galendromus occidentalis* (44%) and the spider *Parasteatoda tepidariorum* (43%) and also for the insects *Locusta migratoria* (45%), *Drosophila melanogaster* (42%) and *Manduca sexta* (47%) to vitellogenin 2 and tick *Ixodes scapularis* (83%), the crab *Limulus polyphemus* (55%) and the oyster *Crassostrea gigas* (55%) to vitellogenin 5. Furthermore, it shows a distinct lipid composition from most arthropod lipoproteins, being composed of 40% free cholesterol, 27% phospholipids, 20% triacylglycerol and 15% hydrocarbons. In addition to binding most hemolymphatic fatty acids, this lipoprotein also binds and transports free cholesterol. In conclusion, the present study provides insight into the macromolecules involved in arachnid metabolism, which have significant potential for future use for the biological control of ticks.

1. Introduction

Brazil is the largest beef exporter and producer in the world. However, the economic impact of ectoparasitosis on this industry has been estimated by Grisi et al., (2002) to be US$ 2.65 billion/year, with the cattle tick *Rhipicephalus microplus* being alone responsible for US$ 2 billion/year (Grisi et al., 2002). Parasitic relations predispose the cattle to myiasis and consequently devalue their leather. Ticks also induce delays in host development and a decrease in milk and meat production and increase the transmission of pathogens, such as *Babesia* spp. and *Anaplasma* spp. (Guimaraes et al., 1998; Heuchert et al., 1999; Horn and Arteche, 1985). Acaricidal chemical control is effective, but for diverse reasons had led to the emergence of acaricide resistance in ticks. The application and misuse of acaricides cause an increase in toxicity to animals and to the environment (Rosario-Cruz et al., 2009; Willadsen, 2006). A rapid increase in the tick population in optimal seasons is promoted by the laying of several hundreds of eggs in a few days (Gonzales, 2003). Thus, an effective strategy to control the tick population could be to interfere with the cycles of egg laying while lowering the larval densities. In most vector ticks, egg development requires a

Abbreviations: CHO, Free Cholesterol; CHOE, Cholesterol Ester; DAG, Diacylglycerol; FFA, Free Fatty Acids; HC, Hydrocarbons; HPTLC, HighPerformance Thin-layer chromatography; Lp, Lipophorin; MAG, Monoacylglycerol; NL, Neutral Lipids; PL, Phospholipids; RmLCP, Rhipicephalus microplus Lipid Carrier Protein; ST, Sterols; TAG, Triacylglycerol

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previous meal of vertebrate blood (Sonenshine, 1992). Therefore, understanding the mechanisms underlying egg development and the role of the host blood components in this process is crucial to identify new strategies to control the proliferation of these arachnids and consequently reduce both disease transmission and economical losses.

Biochemical and molecular biology techniques have been used to clarify essential physiological processes in the cattle tick. The studied processes include heme metabolism (Lara et al., 2005; Lara et al., 2003) and embryogenesis and oogenesis (da Silva Vaz et al., 1998; Logullo et al., 1998; Seixas et al., 2003). Additionally, the vaccines development has emerged as a major strategy for vector control (da Silva Vaz et al., 1998; Leal et al., 2006; Parizi et al., 2011; Seixas et al., 2008). However, there is little information about the lipid transport and metabolism, especially when considering the key role played by such macromolecules for proper tick development (Sonenshine, 1992).

Our knowledge of the predominant hemolymph proteins like lipophorin, storage protein and vitellogenin (Vg) is highly advanced in insects as compared to ticks. In the class Insecta, lipids play a key role in physiological processes, acting as hormones and as key molecules for energy storage. They are crucial under specific high-demand metabolic conditions, such as egg production, metamorphosis and flight activity (Arrese et al., 2001). Lipids are carried through the hydrophilic hemolymphatic environment complexed to a highly specialized apolipoprotein, such as the lipophorin (Lp). Lp transports and distributes different lipid classes to a wide number of target tissues (Chino et al., 1981; Shapiro et al., 1988).

Gudderra et al. (2002) have described carrier proteins in tick species, such as the *Dermacentor variabilis* carrier protein (DvCP) and the *Ornithodoros parkeri* carrier protein (OpCP) (Gudderra et al., 2001). Maya-Monteiro et al., (2000) have described and characterized the main hemelipoprotein (HeLp) in the cattle tick *R. microplus*. However, no work to date has focused on lipid transport and the characterization and validation of the role of lipoproteins, either in *R. microplus* or in any other tick species.

In this study, we describe for the first time the uptake, incorporation, and conversion of simple lipids into complex lipids during the oviposition cycle in the cattle tick. Furthermore, we achieve the purification and biochemical characterization of a new hemolymphatic lipoprotein from a tick. The *R. microplus* lipid-apolipoprotein complex (RmLCP) is shown here for the first time to be responsible for most of the lipid transport in this model.

2. Materials and methods

2.1. Ticks

The ticks used in this study were obtained from a Porto Alegre strain of *R. microplus* free of *Babesia* spp. and were reared on calves raised in a tick-free area and maintained in insulated pens. Calves were infested with 15-day-old tick larvae. After 22 days, fully engorged adult female ticks were collected. The colony is maintained at the Veterinary Faculty from Federal University of Rio Grande do Sul, RS, Brazil. Fully engorged adult females were kept in Petri dishes at 28 °C and 80% relative humidity until the completion of oviposition and death, which took approximately 18 days. The experiments were approved and conducted following the guidelines of the Ethics Committee on Animal Experimentation of UFRGS.

2.2. Hemolymph

Hemolymph of fully engorged female ticks was collected on the fifth day after dropped from the bovine. A needle was used to puncture the dorsal cuticle and gentle pressure was applied to the tick abdomen to release hemolymph. The hemolymph was mixed with a collecting solution (1:1, v/v) containing 0.15 M NaCl, 5 mM EDTA, and protease inhibitors (0.05 mg/mL soybean trypsin inhibitor, 0.05 mg/mL leupeptin, 1 mM benzamidine, and 2.5 mM pepstatin). The solution was centrifuged at room temperature for 5 min at 13,000 × *g*, and the pellet was discarded. The supernatant was stored at −70 °C.

2.3. [9,10-^{3}H] palmitic acid injection in engorged females

1.1.1 [9,10-^{3}H] palmitic acid (PerkinElmer Inc, MA, USA) used for the injection in engorged tick females was diluted in absolute ethanol. Using a 5 µL Hamilton syringe (Hamilton Co, NV, USA), each engorged tick female was injected with 1 µL of tritiated palmitic acid, equivalent to 1 µCi. The injection was always made in the lower left ventral corner, directly into the hemocoel. Control group engorged tick females were injected with absolute ethanol in the same way.

2.4. Liquid scintillation analysis

To assays involving radioactive tritiated palmitic acid, ten microliters of each sample was added in a plastic scintillation Pro Pico vial (PerkinElmer Inc, MA, USA) with 2 mL of liquid scintillation cocktail OptiPhase HiSafe 2 (PerkinElmer Inc, MA, USA) and taken to Tri-Carb 3180TR liquid scintillation analyser (PerkinElmer Inc, MA, USA).

2.5. Distribution of [9,10-^{3}H] palmitic acid among hemolymph proteins

Five minutes after [9,10-^{3}H] palmitic acid injection, the hemolymph of engorged females was collected (as described in Section 2.2). Solid KBr was added to the supernatant to a final concentration of 0.4 g/mL, and the mixture was again centrifuged at 125,000 x *g* in a Beckman ultracentrifuge (Optima L-90 ultracentrifuge, Beckman Coulter, Palo Alto, CA) with a fixed-angle Beckman rotor 50.2 Ti at 4 °C for 20 h. Supernatant fractions were collected from the top. The presence of proteins in each fraction was checked using an SDS-polyacrylamide gel. Fractions containing stained proteins were pooled and extensively dialyzed against PBS. Samples were then concentrated using a vacuum dryer (Savant Speed-Vac®, Thermo Fisher Scientific, MA, USA)) and stored in liquid nitrogen. The protein concentration was estimated by the method of Lowry et al. (1951) using a modified protocol in the presence of 0.5% SDS and using Bovine Serum Albumin (BSA) as the standard. The distribution of proteins and radioactivity along the gradient was analyzed by Polyacrylamide Gel Electrophoresis (PAGE) and scintillation counting (as described in Section 2.4).

2.6. in vivo radiolabeling organs with [9,10-^{3}H] palmitic acid

Radioactive palmitic acid was injected with a Hamilton syringe into the hemocoel (as described in Section 2.3) in 5 groups (1, 5, 15, 30 and 60 min) containing 6 engorged adult females. After that the gut, ovary, fat body and Gene's organ were collected and washing in PBS, homogenized in collect solution (1:1, v/v) containing 0.15 M NaCl, 5 mM EDTA, and protease inhibitors (0.05 mg/mL soybean trypsin inhibitor, 0.05 mg/mL leupeptin, 1 mM benzamidine, and 2.5 mM pepstatin) and then subjected to lipid extraction according to Bligh and Dyer (1959). The injection of radioactive palmitic acid and collection of the organs was done per group so that no overlap occurred over time. The total radioactivity incorporated by each organ collected was analyzed by liquid scintillation counting (as described in Section 2.4).

2.7. Polyacrylamide gel electrophoresis and molecular mass determination

Polyacrylamide slab gels were run under both denaturing (with SDS) (Laemmli, 1970) and nondenaturing conditions (Davis, 1964) to the first fraction of gradient KBr as described in Section 2.5. Electrophoresis was performed at constant voltage (100 V) on Tris-glycine gradient gels of 3–20% acrylamide, and the protein band were stained with Coomassie blue. For molecular mass determinations, the molecular weight standard Rainbow Kit from Amersham (Buckinghamshire,