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In vitro lipid transfer between lipoproteins and midgut-diverticula in the spider *Polybetes pythagoricus*

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ABSTRACT

It has been already reported that most hemolymphatic lipids in the spider *Polybetes pythagoricus* are transported by HDL1 and VHDL lipoproteins. We studied in vitro the lipid transfer among midgut-diverticula (M-diverticula), and either hemolymph or purified lipoproteins as well as between hemolymphatic lipoproteins. M-diverticula and hemolymph were labeled by in vivo ^{14}C-palmitic acid injection. In vitro incubations were performed between M-diverticula and either hemolymph or isolated lipoproteins. Hemolymph lipid uptake was associated to HDL1 (67%) and VHDL (32%). Release from hemolymph towards M-diverticula showed the opposite trend, VHDL 75% and HDL1 45%. Isolated lipoproteins showed a similar behavior to that observed with whole hemolymph. Lipid transfer between lipoproteins showed that HDL1 transfer more ^{14}C-lipids to VHDL than vice versa. Only 38% FFA and 18% TAG were transferred from M-diverticula to lipoproteins, while on the contrary 75% and 73% of these lipids, respectively, were taken up from hemolymph. A similar trend was observed regarding lipoprotein phospholipids. This study supports the hypothesis that HDL1 and hemocyanin-containing VHDL are involved in the uptake and release of FFA, phospholipids and triacylglycerols in the spider *P. pythagoricus*. The data support a directional flow of lipids from HDL1 and VHDL suggesting a mode of lipid transport between lipoproteins and M-diverticula.

1. Introduction

Invertebrate circulating lipoproteins have been studied mainly in the phyla Arthropoda and Mollusca. Lipoproteins of crustaceans, the most studied group of aquatic arthropods, are well known and have been described and reviewed by Lee and Puppione (1978), Chang and O'Connor (1983) and Lee (1991). In terrestrial arthropods, the mechanisms of lipid circulation are well known in insects i.e. Chino (1985) and Soulages and Wells (1994). The occurrence of hemolymphatic lipoproteins in arachnids has been reported in a few species belonging to the orders Araneida, Scorpionida, Solpugida and Acarina (Cunningham et al., 2007). However, lipoproteins were properly characterized in only three species: *Eurypelma californicum*, *Latrodectus mirabilis* and *Polybetes pythagoricus*. Particularly, the hemolymph lipid transport system of *P. pythagoricus* (Araneae: Sparassidae) is composed of three lipoproteins which had been previously characterized: two HDL named HDL1 and HDL2, and one VHDL. HDL1 (2.3 mg of protein/mL hemolymph) is a lipophorin-like lipoprotein regarding size and subunit composition but unlike insect lipophorin, it has mainly phospholipids (PL), free fatty acids (FFA) and triacylglycerols (TAG) (Cunningham et al., 1994). The HDL2 and VHDL (23.6 and 45.4 mg of protein/mL hemolymph) contain hemocyanin as the main apolipoprotein, and PL as the main lipid (Cunningham et al., 1994; Cunningham and Pollero, 1996).

The role of lipoprotein in lipid dynamics is well known only in insect and crustaceans. In insects mainly diacylglycerols (DAG) are transferred from fat body to pre-existing lipophorin particles located in the hemolymph (HL); then these particles may release lipids to other tissues (Blacklock and Ryan, 1994; Soulages and Wells, 1994; Arrese et al., 2001). In crustaceans, lipids are transported in the HL by HDLs (Lee and Puppione, 1978; Chang and O'Connor, 1983); the transfer of different lipids (PL, FFA and TAG) from and to the midgut gland (most important organ regarding lipid metabolism) was demonstrated (García et al., 2002). Little information concerning arachnids is available. Recently, we have reported that HDL1 and VHDL actively participate in the radioactive lipid uptake from midgut-diverticula (M-diverticula), and also showed that radioactivity was taken from HL by M-diverticula, which, in turn synthesized mainly TAG and PL in a ratio close to their lipid class composition (Laino et al., 2009). This organ is quite similar to insect fat body and crustacean midgut gland (sometimes termed hepatopancreas). Spider midgut elaborate branching fills out most of the opisthosoma and is also present in the prosoma surrounding many organs. Two cell types have been described in M-diverticula epithelium: secretory and resorptive cells. Secretory cells contain digestive enzymes, while the resorptive cells have numerous "food vacuoles", process the nutrients further and pass them onto the underlying interstitial tissue or into the hemolymph.

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The role of insect fat body in lipid metabolism has been extensively studied (O'Connor and Gilbert, 1968; Gilbert and Chino, 1974; Arrese et al., 2001). Likewise, crustacean midgut gland is involved in active lipid metabolism and has the combined functions of mammalian liver, intestine, pancreas and adipose tissue (Al-Mohanna et al., 1985; Al-Mohanna and Nott, 1986; González Baró and Pollero, 1988; González Baró et al., 1990; González Baró and Pollero, 1993; García et al., 2002; Lavarías et al., 2006; Lavarías et al., 2007).

In this study we present data about the role of hemolymphatic lipoproteins in the spider *P. pythagoricus*, concerning lipid uptake and release from and to M-diverticula, as well as lipid transfer between lipoproteins. It must be pointed out that the present experiments were carried out in vitro where only donor/acceptor (lipoproteins/M-diverticula) at physiological concentrations participated. Therefore, this study complements previous assays performed in vivo where the design precludes the identification of the organ/s involved in the origin and fate of lipids.

2. Materials and methods

2.1. Biological and chemical materials

Adult specimens of the spider *P. pythagoricus* weighing 2.32 ± 0.5 g were collected from barks of eucalyptus trees 10 km NE from La Plata city, Argentina. Spiders were kept without food in glass jars until used.

All investigations were conducted in compliance with the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals. Studies were approved by a committee of the Consejo Nacional de Investigaciones Científicas y Técnicas. [1-^{14}C]-Palmitic acid (57.0 mCi/mmol) was purchased from Amersham. All chemicals were of analytical grade.

2.2. In vivo labeling with radioactive FA

Radioactive palmitic acid was administered to groups of 5 spiders each. Spiders were maintained at room temperature. Each spider was injected into the leg with 6 μL of an aqueous solution containing 4 μCi (70 nmol) radioactive fatty acid as ammonium salt. A syringe with a needle designed for animal injection (Hamilton Co.) was used. After 1 h incubation, M-diverticula and HL were separated; a labeling distribution similar to that described by Laino et al. (2009) was noted for M-diverticula (45% FFA, 39% TAG, 15% PL) and HL (56% FFA, 16% PL, 28% TAG). These labeled tissues were used for the transfer experiments.

2.3. Lipoproteins and M-diverticula isolation

Animals were cold-anesthetized and HL obtained by severing the spider's legs, and the spider was centrifuged in a tube at low speed (Cunningham et al., 1994).

Aliquots of hemolymph were over layered on a 3 mL NaBr solution (δ = 1.26 g/mL) and centrifuged at 178,000 *g* at 10 °C for 22 h in a Beckman L8 70 M centrifuge with a SW60Ti rotor (Palo Alto, USA). Assuming that the density of spider's hemolymph was 1.006 g/mL, a saline solution of this density was centrifuged simultaneously as blank, and its density was determined in a Bausch & Lomb refractometer. The total volume of the tubes was fractionated from top to bottom into 0.2 mL aliquots. The protein content of each aliquot was monitored spectrophotometrically at 280 nm. Radioactivity in each fraction was measured by liquid scintillation in a Wallac 1214 Rack Beta equipment (Turku, Finland), and density calculated from the control blank tube. Fractions showing an increase in both, A280 and radioactivity (δ = 1.13 and 1.21–1.24 g/mL) were pooled and their protein content determined colorimetrically (Lowry et al., 1951). Radioactive lipid distributions were analyzed as described below.

A dorsal incision was made in the opisthosoma tegument and M-diverticula were carefully dissected out (Laino et al., 2009).

M-diverticula and HL were used for lipid analysis in the experiments of transfer between HL and M-diverticula as well as between isolated lipoprotein.

2.4. Lipid extraction and analysis

Lipids from M-diverticula, HL and isolated lipoproteins were extracted following the procedure of Folch et al. (1957). The general procedure for separation and identification of lipids was described in a previous work (Cunningham and Pollero, 1996).

Total radioactivity was measured by liquid scintillation in a Wallac 1214 Rack Beta equipment. Radioactive lipids were analyzed by chromatography on HPTLC plates (Merck, Germany). Lipids were separated by double development of plates. First, chloroform–methanol–acetic acid–water (65:25:4:4 v/v/v/v) for polar lipid development (half of the plate) followed by a second development with hexane–diethyl ether–acetic acid (80:20:1.5 v/v/v) for neutral lipids until the solvent front reached the top of the plate. Reference lipids used as standards on HP-TLC plates were 1–2 and 1–3 diacylglycerols, cholesterol, tristearin, stearic acid, cholesteryl oleate, a mixture of hydrocarbons, choline and ethanolamine phosphoglycerides. For detection of separated constituents, the plates were exposed to iodine vapors. Radioactivity of each lipid was quantified by liquid scintillation. Each lipid class was identified according to its corresponding standard. Then they were scrapped from the plate, eluted from the silica, and washed three times with chloroform:methanol (2:1) for polar lipids and chloroform:methanol:hexane (2:1:1) for neutral lipids.

2.5. Lipid uptake by hemolymphatic lipoproteins

To explore the lipid uptake by hemolymphatic lipoproteins, labeled M-diverticula (donor) was incubated with unlabeled HL (acceptor). The donor/acceptor ratio was selected taking into account that one found in an individual in physiological condition that is to say 120 ± 0.07 mg M-diverticula with 100 μL HL. Incubations were done in 50 mM potassium phosphate buffer pH 7.4, 0.25 M sucrose, with the addition of 1 μL aprotinin as protease inhibitor in a final volume of 1 mL, similar García et al. (2002). Assays were carried out at 25 °C for 30 min with shaking.

To analyze the behavior of each lipoprotein separately, different incubations under similar conditions to those previously identified were performed: 1) M-diverticula labeled with ^{14}C-lipids with unlabeled HDL1 and 2) M-diverticula labeled with ^{14}C-lipids with unlabeled VHDL. The donor/acceptor ratio was 1 M-diverticula/232.4 μg protein to HDL1 and 1 M-diverticula/4542.7 μg protein to VHDL. Isolated lipoprotein concentrations corresponded to those found in 100 μL of HL (corresponding to an individual).

After incubations, tissue and medium were separated, and M-diverticula were raised with potassium phosphate buffer. Lipoproteins and lipids were extracted and analyzed as described above. All transfer experiments were done at least in triplicate.

2.6. Lipid release from hemolymphatic lipoproteins

To explore the lipid release from the hemolymphatic lipoproteins, labeled lipoproteins (donor) with ^{14}C-lipids were incubated with unlabeled M-diverticula (acceptor). The donor/acceptor ratio was a whole 100 μL HL/M-diverticula (120 ± 0.07 mg), similar to that found in each individual in physiological conditions. Incubations were done in 50 mM potassium phosphate buffer pH 7.4, 0.25 M sucrose, with the addition of 1 μL aprotinin as protease inhibitor in a final volume of 1 mL. Assays were carried out at 25 °C for 30 min, with shaking.

To analyze the behavior of each lipoprotein separately, incubations with labeled isolated lipoproteins under the same conditions to the previous ones were done: 1) labeled HDL1 with unlabeled M-diverticula

and 2) labeled VHDL with unlabeled M-diverticula. The donor/acceptor ratio was under physiological conditions.

After incubations, tissue and medium were separated, and M-diverticula were raised with 50 mM potassium phosphate buffer pH 7.4. Lipoproteins and lipids were extracted and analyzed as described above. All transfer experiments were done at least in triplicate.

2.7. Lipid uptake by hemocytes

In order to elucidate whether hemocytes take up lipids, three spiders were injected with 4 μCi (70 nmol) radioactive fatty acid as ammonium salt into the leg. After 1-h incubation HL were obtained. Hemocytes were isolated by diluting HL in buffer Tris–HCl pH 8 0.1 M, centrifuged at 720 *g* for 10 min, then the pellet was resuspended and centrifuged again at 720 *g* for 30 min. From these hemocytes, lipids were extracted using the Folch et al. (1957) technique and radioactivity was quantified by liquid scintillation counting.

2.8. Lipid transfer between lipoproteins

A constant amount of donor (physiological concentration), labeled HDL1 or VHDL and increasing amounts of acceptor, unlabeled HDL1 or VHDL in an acceptor/donor 0.25, 0.5 and 1 ratio, were incubated in order to study lipid transfer between lipoproteins. The 1 represents 2.3 mg/mL HDL1 and 45 mg/mL VHDL.

These incubations were carried out in 50 mM in phosphate buffer, pH 7.4, volume final 1 mL, at 25 °C for 30 min with shaking.

2.9. Statistical analyses

At least three separate experiments were performed for each study. Samples were analyzed in triplicate and standard deviations were calculated. Data were analyzed by Student's t-test using Instat v 2.0. Results were considered significant at $P<0.05$. Student's t-test was used in each experiment according to the necessary comparisons.

3. Results

3.1. Lipid uptake by lipoproteins

The first set of experiments was designed to determine the lipid uptake by hemolymphatic lipoproteins in whole HL. In order to study this, M-diverticula with radiolabeled lipids were incubated with unlabeled HL. After 30 min incubation, the HL was subjected to density gradient ultracentrifugation, and the radioactivity in each fraction determined as described in Materials and methods. Simultaneous registers of mass and radioactivity showed that the maximum radioactivity was associated to HDL1 and VHDL lipoproteins. HDL2 did not show any associated radioactivity, in agreement with a previous report by Laino et al. (2009). The uptake percentage of labeled lipids in lipoproteins was 28% ± 12 from total label originally associated with M-diverticula, where 67% ± 6 was for HDL1 and 33.7 ± 7 was for VHDL (Fig. 1).

The second set of experiments was done to determine lipid uptake in isolated lipoproteins. In this assay 48% ± 9 and 29% ± 15 of the radioactivity originally associated with M-diverticula was transferred to HDL1 and VHDL, respectively (Fig. 1).

Another set of experiments was performed to assess hemocyte action on the lipid dynamics. ^{14}C-Lipid uptake in hemocytes was analyzed, and only 3% labeling in HL was found to be associated with hemocytes, demonstrating that hemocytes were scarcely involved in transfer assays.

Fig. 2 shows the result of the distribution of labeled lipid classes from experiments in which radioactive M-diverticula were incubated in vitro with unlabeled HL. These experiments evidenced the uptake by lipoproteins of mainly PL, FFA and to a lesser extent TAG. These

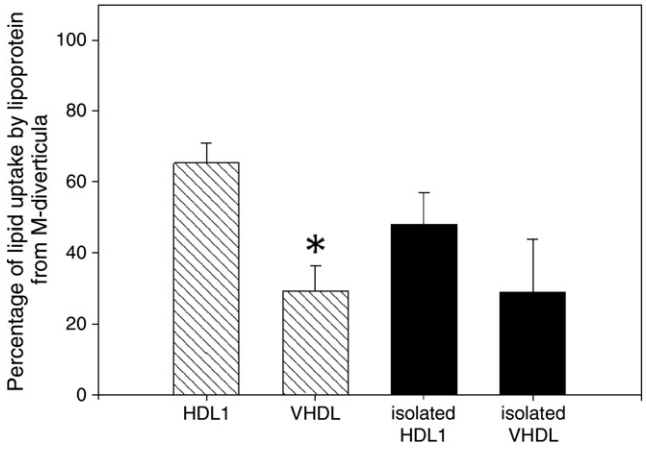

Fig. 1. ^{14}C-Lipid uptake by HL (▨) or isolated lipoproteins (■) after incubation with radiolabelled M-diverticula. Error bars indicate SD of the mean (n = 3). Student's test was used to compare the significance of differences between HDL1 and VHDL in HL and isolated lipoproteins: * $P<0.05$.

experiments also showed the uptake of PL and FFA by both lipoproteins as well as that of most TAG carried out by VHDL.

Fig. 3 displays the relative amount of each lipid class uptake by isolated lipoproteins when incubated in vitro with M-diverticula, being this uptake 57.3% PL, 57.8% FFA an 33.1% TAG for the HDL1, whereas for VHDL this uptake was 38.1%, 56.8% and 32.4% PL, FFA and TAG, respectively. The percentage of uptake was calculated taking into account the content of each lipid in the M-diverticula donor as 100%. Concerning those assays in which the uptake of M-diverticula labeled lipids by isolated lipoproteins was analyzed, it was noted that both lipoproteins uptake PL, FFA and TAG in a similar fashion (around 35–60%) (Fig. 3).

3.2. Lipid release by lipoproteins to M-diverticula

When unlabeled M-diverticula were incubated for 30 min with labeled HL, around 80% of labeling was released to M-diverticula. A similar transfer percentage was observed using isolated lipoproteins as lipid donors, though it may be noted that isolated lipoproteins do not have the same releasing behavior (HDL1 and VHDL, Fig. 4). Thus VHDL has the capacity to release a higher percentage of labeled lipids than HDL1. The percentage of different lipids released by whole HL to M-diverticula is shown in Fig. 5. TAG, FFA and PL were released by 73, 75 and 44%, respectively. Concerning isolated HDL1 and VHDL, TAG,

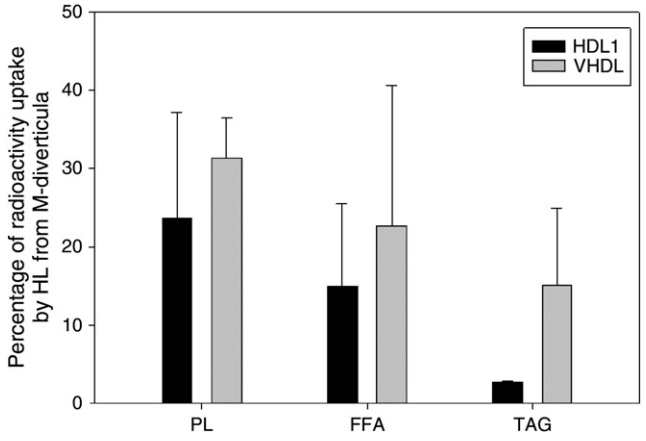

Fig. 2. Percentage of different lipids taken up by lipoproteins (acceptor) from M-diverticula (donor). Assays were carried out using whole hemolymph, and then each lipoprotein analyzed separately. (Values represent the average of three experiments ± SD) ■ HDL1; ■ VHDL.

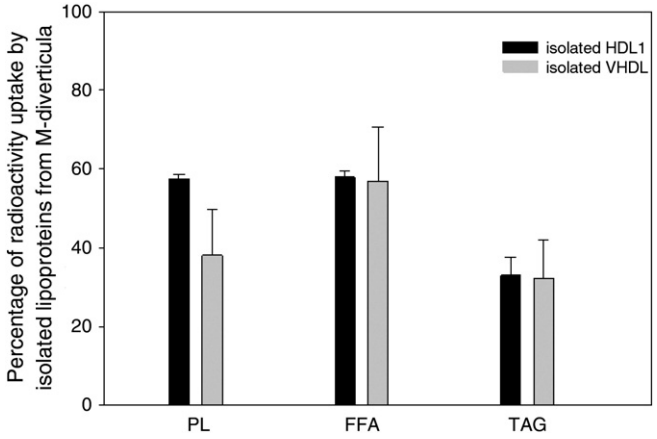

Fig. 3. Percentage of different lipids taken up by lipoproteins (acceptor) from M-diverticula (donor). Assays were carried out with isolated lipoproteins. (Values represent the average of three experiments ± SD). ■ HDL1 ■ VHDL.

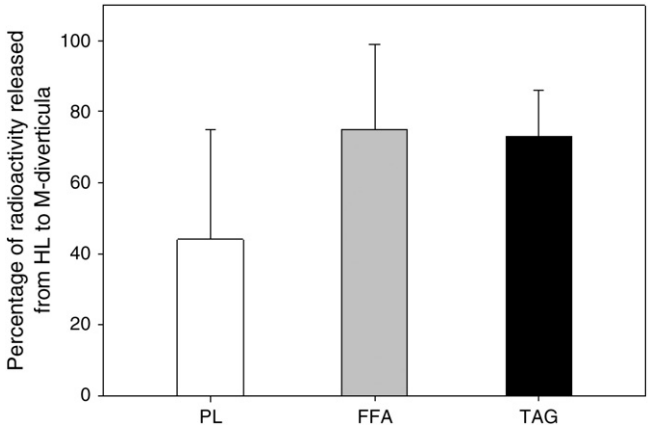

Fig. 5. ^{14}C-lipid classes released by lipoproteins (donor) to M-diverticula (acceptor). Assays were carried out with whole HL. (Values are expressed as % and represent the average of three experiments ± SD).

FFA and PL were transferred in a similar percentage (around 40–70%) (Fig. 6).

Fig. 7 shows the transfer of ^{14}C-lipids between VHDL and HDL1 and vice-versa in incubations at different acceptor/donor ratios. This experiment was performed in the presence of a constant amount of lipid-donor (^{14}C-VHDL or ^{14}C-HDL1) at increasing acceptor levels (unlabeled HDL1 or VHDL) and expressed as % transfer radioactivity. After incubation, lipoproteins were subjected to gradient density ultracentrifugation, and radioactivity was measured in each fraction (inset Fig. 7). When HDL1 was used as lipid-donor, 60% lipids were transferred from HDL1 to VHDL. On the other hand, when VHDL was used as lipid-donor in the presence of increasing HDL1 levels the transfer was around 20%. These results demonstrated that HDL1 is capable of transferring more lipids-^{14}C to VHDL than from this one to HDL1.

4. Discussion

In earlier studies we have reported the lipid–protein composition and the physicochemical properties of the three circulating lipoproteins (HDL1, HDL2 and VHDL) in the spider *P. pythagoricus* (Cunningham et al., 1994; Cunningham and Pollero, 1996; Cunningham et al., 2007). We have speculated about the functionality of these three lipoproteins on the basis of their lipid and protein composition.

In the present study the metabolism of ^{14}C-palmitic, known in this spider from previous studies, was utilized to generate radiolabeled lipids (FFA, PL, and TAG) in both, hemolymph lipoproteins and M-diverticula which play a crucial role in the circulation and synthesis and storage of lipids, respectively (Laino et al., 2009). The use of these labeled tissues with the unlabeled ones, at physiological concentrations allowed us to determine the lipid uptake and transfer by lipoproteins in a closed system, selecting a M-diverticula and HL ratio similar to those present in a spider.

As expected, around 20% uptake of labeling present in M-diverticula was performed by HDL1 and VHDL. As previously observed, HDL2 was not involved in the lipid dynamics. As discussed by Laino et al. (2009) the main function of HDL2 would be O_2 transport through its hemocyanin subunit and its lipid may probably only play a structural lipid function, a stabilizing role also reported for crustacean hemocyanin (Zatta, 1981).

Concerning the other two lipoproteins, the uptake of most radiolabeled lipids was carried out by HDL1 when compared to VHDL. This was expected as HDL1 contains around 30% lipids whereas VHDL only has 3%. Besides, in other arthropod groups lipoproteins with this hydration density are responsible for most of the lipid transport and are the predominant lipoprotein in insects (Gonzalez et al., 1995) and in crustaceans (Yepiz-Plascencia et al., 2000). Though data concerning arachnid lipoproteins is still incomplete and disperse, the available data suggest that HDL perform this function. For instance, *L. mirabilis* has two lipoproteins, one of them the HDL1 has

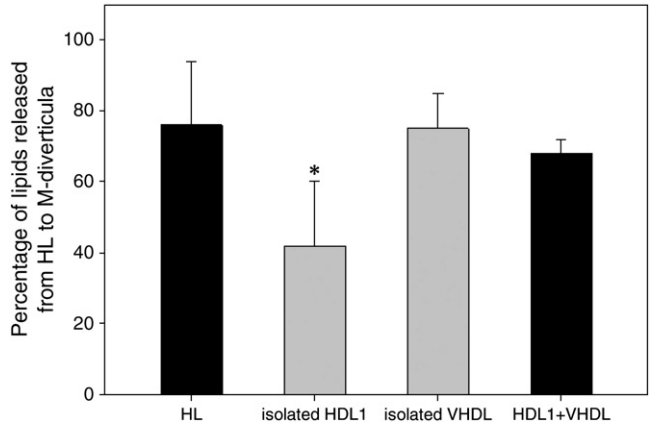

Fig. 4. ^{14}C-lipids release to M-diverticula (acceptor) by whole HL (donor) (■), isolated lipoproteins (■) and HDL1 + VHDL (■). Error bars indicate SD of the mean (n = 3). Student's test was used to compare the significance of the differences between isolated HDL1 and isolated VHDL: * $P<0.05$.

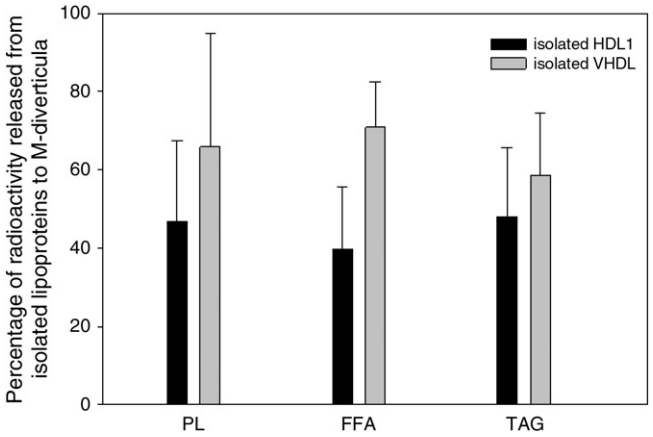

Fig. 6. ^{14}C-lipid classes released by lipoproteins (donor) to M-diverticula (acceptor). Assays were carried out with isolated lipoproteins. (Values are expressed as % and represent the average of three experiments ± SD). ■ HDL1 and ■ VHDL.

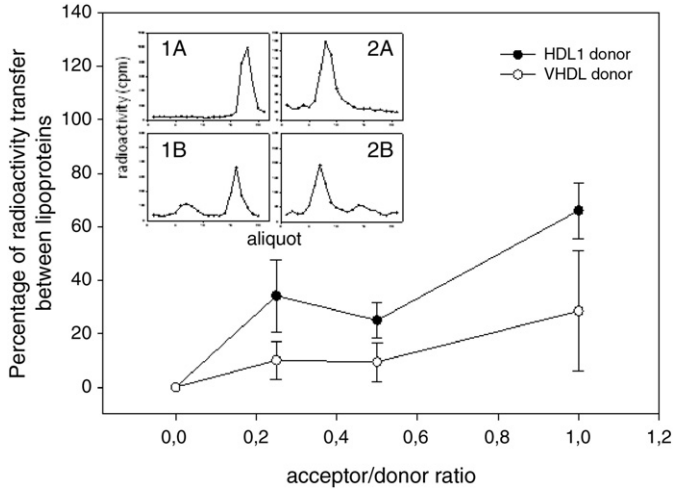

Fig. 7. ^{14}C-lipid transfer between lipoproteins at different acceptor/donor ratios. –●– HDL1 donor, –○– VHDL donor. Inset: the lipoproteins were subjected to density gradient ultracentrifugation followed by fractionation (0.2 mL) and determination of the radioactivity in each fraction. (panel 1A) VHDL—^{14}C donor, (panel 1B) VHDL—^{14}C (donor) with HDL1 unlabeled (acceptor) ratio 1/1, (panel 2A) HDL1—^{14}C donor, (panel 2B) HDL1—^{14}C (donor) with VHDL unlabeled (acceptor) ratio 1/1.

the same density (1.13 g/mL) to that of *P. pythagoricus*, and it carries 80% of the total hemolymphatic lipids (Cunningham et al., 2000).

Arthropod hemocyanins are multifunctional particles. Its main function is the O_2 transport, but have other functions such as buffer and osmolites (Paul and Pirow, 1998), hormonal transport during molting (Jaenicke et al., 1999) as well as phenoloxidase activity (Decker and Rimke, 1998). It was demonstrated in in vivo experiments that *P. pythagoricus* VHDL (a lipoprotein also containing hemocyanin) is involved in the lipid transport and uptake from M-diverticula (Laino et al., 2009). However, the design of those in vivo studies precluded the description of lipoprotein behavior in the lipid dynamics. The present work clearly shows the contribution of VHDL to spider lipid dynamics either isolated or as a HL component. Results indicate that it plays an important role in the uptake, though not predominant, and in the lipid release (predominant) to M-diverticula, as it releases 30% more labeling than HDL1. There is an apparent controversy between HDL1 and VHDL in their relative importance in the lipid uptake and release since the uptake of most lipids is performed by HDL1, but most lipids are released to the M-diverticula by VHDL; this fact may be explained by lipid transfer between both lipoproteins. This leads to the following hypothesis: the uptake of most lipids is carried out by HDL1 as reported by Laino et al. (2009), then transferred while circulating in HL to VHDL (Fig. 7) as reported in the insect *Triatoma infestans* (Gonzalez et al., 1995). Lastly, VHDL would release lipids to other tissues like muscle (Laino et al., 2009) or M-diverticula (Fig. 4). In vivo experiments carried out by Laino et al. (2009) further support this hypothesis as they have demonstrated that VHDL releases more lipids than HDL1 to the different tissues.

The function of hemocyanin as a lipid transport particle has been analyzed in invertebrates, mainly in the mollusc *Octopus tehuelchus* (Heras and Pollero, 1990), and suggested in the crustacean *Pacifastacus leniusculus* (Hall et al., 1995). In molluscs, hemocyanin is the apolipoprotein of a VHDL named LP3, which mainly transports FFA, hydrocarbons and esterified sterols. PL are associated to LP1 (Heras and Pollero, 1990), which has been also demonstrated to contain hemocyanin (Heras and Pollero, 1992). In addition, there are insect lipoproteins where HDLp transports DAG and VHDL transports FFA (Rimoldi et al., 1989; Gonzalez et al., 1991; Gonzalez et al., 1995). Our present results showed that in spider lipoproteins (HDL1 and VHDL), the role in the dynamics of FFA, PL and TAG transfer is quite similar.

Spiders and molluscs have different ways of transporting lipids, though both of them share similar lipids classes FFA and PL as the major lipids transported. The evolution of both groups shows they have diverged at the Cambrian age. As a result, molluscs and arthropods contain hemocyanins with different amino acid sequences. Moreover, the role of hemocytes in HL lipid uptake is also different in both groups: in cephalopods, the uptake of 30% lipids is done by hemocytes, but only 3% is found associated with hemocytes in *P. pythagoricus*. The hemocyanin role in the lipid transport cannot be generalized for all spiders, as its association with lipids in tarantula could not be established (Stratakis et al., 1993). The same fact could be ascribed to molluscs where hemocyanin behaves as an apolipoprotein in *O. tehuelchus*, (Heras and Pollero, 1992) but not in the gastropod *Pomacea canaliculata* (Garín and Pollero, 1995). More research is necessary to elucidate the functional–structural relationships throughout hemocyanin evolution in different groups of invertebrates.

In vitro experiments were carried out for a short time in order to avoid the possibility of lipid synthesis within the M-diverticula masking the transfer results. On the other hand, in experiments in vivo it was corroborated that the metabolism of acylglycerols at short times, is scarce (Laino et al., 2009).

Concerning lipid circulation, it is evident that lipoproteins tend to uptake mainly PL and FFA from M-diverticula, in agreement with their lipid composition, as HDL1 and VHDL, contain around 60% PL and 15% FFA. The PL were found to be the predominant circulating lipids in spiders (Cunningham et al., 2007) and crustaceans (Lee and Puppione, 1988; García et al., 2002). These lipids would then be distributed to different tissues. Both HL lipoproteins mainly transfer FFA that would be employed for the synthesis of acylglycerols like in the insect *Rhodnius prolixus* (Atella et al., 2000) or the crustacean *Macrobrachium borellii* (García et al., 2002). TAG stored in M-diverticula are mostly used as energy source as previously observed in *P. pythagoricus* (Laino et al., 2009) in agreement with the observed low transfer to lipoproteins.

In conclusion, this study supports the hypothesis that HDL1 and hemocyanin-containing VHDL are involved in the uptake and release of FFA, PL and TAG in the spider *P. pythagoricus*. The study supports a directional flow of lipids from HDL1 and VHDL suggesting a novel mode of lipid transport between lipoproteins and M-diverticula.

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References

Al-Mohanna, S.Y., Nott, J.A., 1986. B-Cells and digestion in the hepatopancreas of *Penaeus semisulcatus* (Crustacea: Decapoda). J. Mar. Biol. Assoc. UK 66, 403–414.
Al-Mohanna, S.Y., Nott, J.A., Lane, D.J.W., 1985. Mitotic E- and secretory F-cells in the hepatopancreas of the shrimp *Penaeus semisulcatus* (Crustacea: Decapoda). J. Mar. Biol. Assoc. UK 65, 901–910.
Arrese, E.L., Canavoso, L.E., Jouni, Z.E., Pennington, J.E., Tsuchida, K., Wells, M.A., 2001. Lipid storage and mobilization in insects: current status and future directions. Insect Biochem. Mol. Biol. 31, 7–17.
Atella, G.C., Arruda, M.A., Masuda, H., Gondim, K.C., 2000. Fatty acid incorporation by *Rhodnius prolixus* midgut. Arch. Insect Biochem. Physiol. 43, 99–107.
Blacklock, B.J., Ryan, R.O., 1994. Hemolymph lipid transport. Insect Biochem. Mol. Biol. 24, 855–873.
Chang, E.S., O'Connor, J.M., 1983. Metabolism and transport of carbohydrates and lipid. In: Mantel, L.H. (Ed.), The Biology of Crustacea. Academic Press, New York, pp. 263–278.
Chino, H., 1985. Lipid transport: biochemistry of hemolymph lipophorin. In: Kerkut, G.A., Gilbert, L.I. (Eds.), Comprehensive Insect Physiology, Biochemistry and Pharmacology, Vol. 10. Pergamon Press, New York, pp. 115–135.
Cunningham, M.L., García, F., Pollero, R.J., 2007. Arachnid lipoproteins: Comparative aspects. Comp. Biochem. Physiol. C 146, 79–87.
Cunningham, M.L., Gonzalez, A., Pollero, R.J., 2000. Characterization of lipoproteins isolated from the hemolymph of the spider *Latrodectus mirabilis*. J. Arachnol. 28, 49–55.

Cunningham, M.L., Pollero, R.J., 1996. Characterization of lipoprotein fractions with high content of hemocyanin in the hemolymphatic plasma of *Polybetes pythagoricus*. J. Exp. Zool. 274, 275–280.

Cunningham, M.L., Pollero, R.J., Gonzalez, A., 1994. Lipid circulation in spiders. Transport of phospholipids, free acids and triacylglycerols as the major lipid classes by a high-density lipoprotein fraction isolated from plasma of *Polybetes pythagoricus*. Comp. Biochem. Physiol. B 109, 333–338.

Decker, H., Rimke, T., 1998. Tarantula hemocyanin shows phenoloxidase activity. J. Biol. Chem. 273, 25889–25892.

Folch, J., Lees, M., Sloane-Stanley, G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497–509.

García, F., González Baró, M.R., Pollero, R.J., 2002. Transfer of lipids between hemolymph and hepatopancreas in the shrimp *Macrobrachium borellii*. Lipids 37, 581–585.

Garín, C.F., Pollero, R.J., 1995. Isolation and characterization of a low density lipoprotein fraction from plasma of the aquatic snail *Ampullaria canaliculata*. Comp. Biochem. Physiol. B 111, 147–150.

Gilbert, L.I., Chino, H., 1974. Transport of lipids in insects. J. Lipid Res. 15, 439–456.

González Baró, M.R., Irazú, C.E., Pollero, R.J., 1990. Palmitoyl-CoA ligase activity in hepatopancreas and gill microsomes of the freshwater shrimp *Macrobrachium borellii*. Comp. Biochem. Physiol. B 97, 129–133.

González Baró, M.R., Pollero, R.J., 1988. Lipid characterization and distribution among tissues of the freshwater crustacean *Macrobrachium borellii* during an annual cycle. Comp. Biochem. Physiol. B 91, 711–715.

González Baró, M.R., Pollero, R.J., 1993. Palmitic acid metabolism in hepatopancreas of the freshwater crustacean *Macrobrachium borellii* during an annual cycle. Comp. Biochem. Physiol. B 106, 71–75.

Gonzalez, M.S., Rimoldi, O., Brenner, R., 1995. Studies on very-high-density lipoprotein of *Triatoma infestans* hemolymph in relation to its function as free fatty acid carrier. Comp. Biochem. Physiol. B 110, 767–775.

Gonzalez, M.S., Soulages, J.L., Brenner, R.R., 1991. Changes in the hemolymph lipophorin and very high density lipoprotein levels during the 5th nymphal and adult stages of *Triatoma infestans*. Insect Biochem. 21, 679–687.

Hall, M., vanHeusden, M.C., Söderhäll, K., 1995. Identification of the major lipoproteins in crayfish hemolymph as proteins involved in immune recognition and clotting. Biochem. Biophys. Acta 22, 939–946.

Heras, H., Pollero, R.J., 1990. Occurrence of plasma lipoproteins in octopods. Partial characterization and interorgan transport of lipids. J. Exp. Mar. Biol. Ecol. 140, 29–38.

Heras, H., Pollero, R.J., 1992. Hemocyanin as an apolipoprotein in the hemolymph of the cephalopod *Octopus tehuelchus*. Biochim. Biophys. Acta 1125, 245–250.

Jaenicke, E., Foll, R., Decker, H., 1999. Spider hemocyanin binds ecdysone and 20-OH-ecdysone. J. Biol. Chem. 274, 34267–34271.

Laino, A., Cunningham, M.L., Garcia, F., Heras, H., 2009. First insight into the lipid uptake, storage and mobilization in arachnids: role of midgut diverticula and lipoproteins. J. Insect Physiol. 55, 1118–1124.

Lavarías, S., García, F., Pollero, R.J., Heras, H., 2007. Effect of the water-soluble fraction of petroleum on microsomal lipid metabolism of *Macrobrachium borellii* (Arthropoda: Crustacea). Aquat. Toxicol. 82, 265–271.

Lavarías, S., Pollero, R.J., Heras, H., 2006. Activation of lipid catabolism by the water-soluble fraction of petroleum in the crustacean *Macrobrachium borellii*. Aquat. Toxicol. 77, 190–196.

Lee, R.F., 1991. Lipoproteins from the hemolymph and ovaries of marine invertebrates. In: Gilles, R. (Ed.), Advances in Comparative and Environmental Physiology, Vol. 7. Springer-Verlag, London, pp. 187–208.

Lee, R.F., Puppione, D.L., 1978. Serum lipoproteins in the spiny lobster, *Panulirus interruptus*. Comp. Biochem. Physiol. B 59, 239–243.

Lee, R.F., Puppione, D.L., 1988. Lipoproteins I and II from the hemolymph of the Blue Crab *Callinectes sapidus*: lipoprotein II associated with vitellogenesis. J. Exp. Zool. 248, 278–289.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., Randall, R., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

O'Connor, J.D., Gilbert, L.I., 1968. Aspects of lipid metabolism in crustaceans. Am. Zool. 8, 529–539.

Paul, R., Pirow, R., 1998. The physiological significance of respiratory proteins in invertebrates. Zoology 100, 319–327.

Rimoldi, O.J., Soulages, J.L., Gonzalez, S.M., Peluffo, R.O., Brenner, R.R., 1989. Purification and properties of the very high density lipoprotein from the hemolymph of adult *Triatoma infestans*. J. Lipid Res. 30, 857–864.

Soulages, J.L., Wells, M.A., 1994. Lipophorin: the structure of an insect lipoprotein and its role in lipid transport in insects. Adv. Protein Chem. 45, 371–415.

Stratakis, E., Fragkiadakis, G., Tentes, I., 1993. Purification and properties of the fatty acid-binding VHDL from the hemolymph of the spider *Eurypelma californicum*. J. Exp. Zool. 267, 483–492.

Yepiz-Plascencia, G., Vargas-Albores, F., Higuera-Ciapara, I., 2000. Penaeid shrimp hemolymph lipoproteins. Aquaculture 191, 177–189.

Zatta, P., 1981. Protein-lipid interactions in *Carcinus maenas* (Crustacea) hemocyanin. Comp. Biochem. Physiol. B 69, 731–735.