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To complete its replication cycle, a shrimp virus changes the population of long chain fatty acids during infection via the PI3K-Akt-mTOR-HIF1α pathway

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ABSTRACT

White spot syndrome virus (WSSV), the causative agent of white spot disease (WSD), is a serious and aggressive shrimp viral pathogen with a worldwide distribution. At the genome replication stage (12 hpi), WSSV induces a metabolic rerouting known as the invertebrate Warburg effect, which boosts the availability of energy and biosynthetic building blocks in the host cell. Here we show that unlike the lipogenesis that is seen in cancer cells that are undergoing the Warburg effect, at 12 hpi, all of the long chain fatty acids (LCFAs) were significantly decreased in the stomach cells of WSSV-infected shrimp. By means of this non-selective WSSV-induced lipolysis, the LCFAs were apparently diverted into β-oxidation and used to replenish the TCA cycle. Conversely, at 24 hpi, when the Warburg effect had ceased, most of the LCFAs were significantly up-regulated and the composition was also significantly altered. In crayfish these changes were in a direction that appeared to favor the formation of WSSV virion particles. We also found that, at 24 hpi, but not at 12 hpi, the PI3K-Akt-mTOR-HIF1α pathway induced the expression of fatty acid synthase (FAS), an enzyme which catalyzes the conversion of acetyl-CoA into LCFAs. WSSV virion formation was impaired in the presence of the FAS inhibitor C75, although viral gene and viral DNA levels were unaffected. WSSV therefore appears to use the PI3K-Akt-mTOR pathway to induce lipid biosynthesis at 24 hpi in order to support viral morphogenesis.

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1. Introduction

The Warburg effect is defined as aerobic glycolysis, and it is characterized by enhanced glucose uptake, glycolysis and lactate fermentation (Warburg, 1956; Dang, 2012). This kind of metabolic reprogramming is frequently seen in cancer cells, and it is used to provide energy and metabolic intermediates for successful proliferation (Dang, 2012; Menendez and Lupu, 2007; Deberardinis et al., 2008; Vander Heiden et al., 2009; Baenke et al., 2013). In cancer cells that are undergoing the Warburg effect, these energy requirements and necessary intermediates are also supplied by the reactivation of *de novo* lipogenesis (Baenke et al., 2013; Mashima et al., 2009) and the uptake of lipids from the tissue environment (Kuemmerle et al., 2011). The fatty acids and other lipid materials that are generated by these processes are essential substrates for energy metabolism (Menendez and Lupu, 2007; Mashima et al., 2009) as well as the major components of all biological lipid membranes (Menendez and Lupu, 2007; Vander Heiden et al., 2009; Mashima et al., 2009).

The Warburg Effect is also seen in vertebrate cells infected by oncogenic viruses such as KSHV (Kaposi's sarcoma-associated herpesvirus) (Delgado et al., 2010) and HCMV (Human cytomegalovirus) (Munger et al., 2008), and a recent paper (Chen et al., 2011) further showed that it occurs in shrimp hemocytes that are infected by the White Spot Syndrome Virus (WSSV). WSSV, which is a large enveloped (approximately 300 kbp) dsDNA invertebrate virus with

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an *in vivo* replication cycle of 22–24 h, was found to trigger the Warburg effect at the WSSV genome replication stage (12 hpi [hours post infection]) via the activation of the PI3K-Akt-mTOR pathway (Su et al., 2014). This pathway also mediates the Warburg effect in cancer cells (Deberardinis et al., 2008; Vander Heiden et al., 2009; Robey and Hay, 2009; Bhatt et al., 2012) and in vertebrate cells infected by oncogenic viruses (Street et al., 2004; Mannová and Beretta, 2005; Guo et al., 2007; Peng et al., 2010; Martin et al., 2012; Bhatt and Damania, 2013), and it also involved in lipogenesis.

In the present paper, we look more closely at the changes in lipid metabolism in WSSV-infected shrimp. In the first part of this study, we used a previously constructed database to identify and measure the expression levels of proteins involved in TAG (triacylglycerols)/FA (fatty acid) metabolism in shrimp hemocytes at 12 and 24 hpi. This database was compiled from results obtained by liquid chromatography tandem mass spectrometry (LC-MS/MS) as described in our study (Su et al., 2014). We also used gas chromatography–mass spectrometry (GC/MS) to monitor global lipodomic changes in long chain fatty acids (LCFAs) induced by WSSV in shrimp stomachs at the same time points. (Stomachs were used instead of hemocytes because a much longer amount of material was required for each lipidomics sample.) In the second part of this study, to investigate the possible use of fatty acids in virion morphogenesis, we used lipidomic analysis to compare the compositions of the fatty acids in the virion particle and in the stomach of the host animal (crayfish). Lastly, we investigated whether the PI3k-Akt-mTOR pathway is involved in the WSSV-induced lipid changes. Based on our results, we propose a model of how WSSV regulates the lipid balance to its advantage during the two stages of the viral replication cycle.

2. Materials and methods

2.1. Experimental animals and virus inoculum

The *Litopenaeus vannamei* (~3 g body weight) used in this study were purchased from the Aquatic Animal Center, National Taiwan Ocean University (NTOU). The experimental shrimp were maintained for 1–2 days in water tank systems containing sterilized seawater (30 ppt at 25–27 °C). The virus used in this study was white spot syndrome virus (WSSV) (Taiwan isolate, GenBank accession no. AF440570). To prepare the WSSV inoculum stock, hemolymph was collected from moribund shrimp that had been infected by the WSSV Taiwan isolate and subjected to centrifugation (10,000× *g*). The supernatant was diluted with PBS (phosphate-buffered saline) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) and stored at −80 °C. The experimental inoculum was prepared from this stock by dilution (10^{-4}) with PBS. The shrimp were injected with WSSV inoculum (100 μl/shrimp) by intramuscular injection. The challenge titer was such that at 3 days post challenge, the mortality was 50%, increasing to 100% at 5 days post injection.

2.2. Using our database of liquid chromatography tandem mass spectrometry (LC-MS/MS) results to analyze proteins involved in TAG (triacylglycerols)/FA (fatty acids) metabolism in WSSV-infected hemocytes

In a previous study (Su et al., 2014), we used LC-MS/MS to analyze the proteome of WSSV-infected hemocytes. These results were subsequently compiled into a database, and this was used here to analyze the proteins involved in TAG/FA metabolism. The shrimp used in our previous study were injected with PBS or WSSV, and 3–5 pooled hemocyte samples (5 shrimp in each sample) were collected from each group with an anticoagulant (450 mM NaCl, 10 mM KCl, 10 mM EDTA, 10 mM Tris–HCl, pH 7.5) at 12 and 24 hpi. The methods for hemocyte protein extraction, experimental procedures and data evaluation for LC-MS/MS label-free quantitative proteomic analysis are all described in full detail in our previous study (Su et al., 2014).

2.3. Preparation of WSSV-infected shrimp organs for lipidomic analysis and gene expression

After Torin 1 (Tocris Bioscience) was dissolved in dimethyl sulfoxide (DMSO) to provide a stock solution, this stock was diluted with PEG solvent (0.25% polyethylene glycol, 0.25% Tween 20 and 0.15 M NaCl). Shrimp were pretreated with PEG or Torin 1 (20 μg/g shrimp) by intramuscular injection 2 h before being challenged with WSSV and PBS to produce a total of four experimental groups: the PEG/PBS group, the PEG/WSSV group, the TR1/PBS group and the TR1/WSSV group. At 12 and 24 hpi, 5–6 pooled samples were collected (10 shrimp in each pool). Stomach samples were used for the lipodomics, while hemocyte and pleopod were collected for gene expression and the detection of viral copy number, respectively. (Stomachs were used for the lipidomics assays because the amount of lyophilized material required (50 mg) equates to ~5000 shrimp in each pooled sample if hemocytes here used as the same tissue. Although it is not ideal to use stomach cells as a proxy for hemocytes, this should nevertheless be acceptable as we have found that WSSV alters the metabolic intermediates related to aerobic glycolysis in a comparable way in these two cell types [unpublished data].)

2.4. Preparation of WSSV-infected crayfish stomachs and WSSV virions for lipidomic analysis

Crayfish (*Procambarus clarkia* obtained from a commercial culture farm in Pingtung, Taiwan) were used for this part of the study because they can sustain a heavier WSSV virus load than shrimp, and thus ensure that sufficient amounts of WSSV virion particles could be collected for lipodomic analysis. To prepare the virus inoculum, hemolymph collected from WSSV-infected moribund crayfish was centrifuged to remove the hemocytes and then kept at −80 °C before use. Crayfish were injected with diluted virus stock (1: 500 in PBS). The titer was such that 1 week post challenge, the mortality was 50%, increasing to 100% 1 week later (2 weeks post challenge). At 1 week post challenge, stomach tissue was collected while WSSV virion particles were purified from other tissues (except for the organs in the cephalothorax) based on the methods described by Xie et al. (2005) and Tsai et al. (2006). The purity of the WSSV virion samples was checked using SDS-PAGE, Western blotting, negative staining and TEM (Transmission Electron Microscopy).

2.5. Using gas chromatography–mass spectrometry (GC/MS) to analyze the lipidome of WSSV-infected shrimp stomachs and the fatty acid profiles of WSSV-infected crayfish stomachs and WSSV virion particles

The fatty acids were exhaustively extracted from homogenized shrimp and crayfish stomach tissue samples (~50 mg per sample) and from the purified WSSV virion particles (~80 mg per sample) in 10 mL of a 2:1 (v/v) chloroform/methanol mixture after the addition of 50 μl of a chloroform solution containing 0.5 mg C22:0 fatty acid (Sigma–Aldrich) (C22:0 is not found in shrimp stomachs and it was used here as an internal standard). After overnight shaking, debris was removed from each mixture by filtration, and the liquid containing the fatty acid extracts was dried at 40 °C using a rotary

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