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Silencing of HIF-1 in WSSV-infected white shrimp: Effect on viral load and antioxidant enzymes

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ABSTRACT

Hypoxia inducible factor-1 (HIF-1) is a transcriptional factor that induces genes involved in glucose metabolism. HIF-1 is formed by a regulatory α-subunit (HIF-1α) and a constitutive β-subunit (HIF-1β). The white spot syndrome virus (WSSV) induces a shift in glucose metabolism and oxidative stress. HIF-1α is associated with the induction of metabolic changes in tissues of WSSV-infected shrimp. However, the contributions of HIF-1 to viral load and antioxidant responses in WSSV-infected shrimp have been not examined. In this study, the effect of HIF-1 silencing on viral load and the expression and activity of antioxidant enzymes (superoxide dismutase-SOD, glutathione S-transferase-GST, and catalase) along with oxidative damage (lipid peroxidation and protein carbonyl) in tissues of white shrimp infected with the WSSV were studied. The viral load increased in hepatopancreas and muscle after WSSV infection, and the accumulative mortality was of 100% at 72 h post-infection. The expression and activity of SOD, catalase, and GST decreased in each tissue evaluated after WSSV infection. Protein carbonyl concentrations increased in each tissue after WSSV infection, while lipid peroxidation increased in hepatopancreas, but not in muscle. Silencing of HIF-1α decreased the WSSV viral load in hepatopancreas and muscle of infected shrimp along with shrimp mortality. Silencing of HIF-1α ameliorated the antioxidant response in a tissue-specific manner, which translated to a decrease in oxidative damage. These results suggest that HIF-1 is essential for restoring the antioxidant response, which counters the oxidative injury associated with WSSV infection.

1. Introduction

Hypoxia inducible factor-1 (HIF-1) is a transcriptional factor formed by a regulatory α-subunit (HIF-1α) that regulates genes involved in glucose metabolism (Semenza, 1998, 1999). In the white shrimp *Litopenaeus vannamei*, HIF-1α subunits is induced during hypoxia in a tissue-specific manner (Soñanez-Organis et al., 2009) and their silencing demonstrated that key glycolytic genes (hexokinase-HK, phosphofructokinase-PFK, fructose 1,6-bisphosphatase-FBP, lactate dehydrogenase-LDH) are regulated via HIF-1 pathway during hypoxia (Cota-Ruiz et al., 2016; Soñanez-Organis et al., 2010, 2011, 2012).

The White Spot Syndrome Virus (WSSV) (genus *Whispovirus*, family *Nimaviridae*) is a double-stranded DNA virus that causes 100% accumulated mortality within 10 days of infection on shrimp farms (van Hulten et al., 2001; Yang et al., 2001). WSSV induces metabolic shifts (Chen et al., 2011; Hernández-Palomares et al., 2018; Su et al., 2014) and oxidative stress from reactive oxygen species (ROS) production (Liu et al., 2010; Mohankumar and Ramasamy, 2006) in infected shrimp. ROS are highly reactive and unstable molecules that contribute to the chemical defense against pathogens. Overproduction of ROS or a deficiency in the antioxidant defense mechanisms may lead to oxidative stress that, in addition to causing cellular damage, may limit the virus replication during infection of the host (Schwarz, 1996; Valyi-Nagy and Dermody, 2005). Cells have antioxidants defenses mechanisms to balance ROS overproduction and oxidative stress, which includes non-enzymatic molecules (e.g. glutathione, vitamins, and uric acid) and enzymes (e.g. superoxide dismutase-SOD, catalase, glutathione S-transferase-GST and glutathione peroxidase (GPx) (Blokhina et al.,

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2003; Nordberg and Arner, 2001; Valko et al., 2007).

In shrimp hemocytes WSSV induced aerobic glycolysis for successful viral replication, restored host redox balance, and countered ROS production via the phosphatidylinositol 3 kinase/Akt/mammalian target of rapamycin (PIK3-Akt-mTOR) pathway (Chen et al., 2011; Chen et al., 2016; Su et al., 2014). Recently, we demonstrated that HIF-1α is upregulated during the WSSV infection contributing to the induction of LDH expression and glucose metabolism in energetically active tissues in shrimp (Hernández-Palomares et al., 2018). However, the effect of HIF-1 silencing on viral load along with the antioxidant response has not been examined. In this work, we report the tissue-specific effect of HIF-1 silencing over the viral load, along with the expression and activity of antioxidant enzymes (SOD, catalase, and GST) and oxidative damage (protein carbonyl and lipid peroxidation) in WSSV-infected shrimp.

2. Materials and methods

2.1. Silencing of HIF-1 and WSSV infection

Juvenile *L. vannamei* shrimp (15 ± 2 g) were obtained from a shrimp farm located in the state of Sonora, México, and acclimated during four weeks to controlled temperature (between 28 °C and 30 °C), salinity (35 ppt), recirculating-water, aeration, and were fed to 3% of their biomass with camaronina 35 (Purina). Seventy healthy shrimp and free of specific pathogens were used for this study. The inoculum of WSSV was obtained by homogenizing the muscle of WSSV-infected moribund shrimp in saline buffer (20 mM Tris, 400 mM NaCl; pH = 7.4). The homogenate was centrifuged at 10,000 rpm (9 min 4 °C) and the supernatant was filtered using sterile filters (0.8 μm pore). The experimental inoculum was adjusted to 141, 416 copy number of WSSV/100 μL such that the cumulative mortality reached approximately 100% between 2 and 3 days after WSSV exposure.

The double-stranded RNA (dsRNA) for HIF-1α was synthezied from the regions corresponding to the positions 200–1036 of their homology for *L. vannamei* nucleotide sequence (GenBank accession number FJ807918) using the RiboMAX® Large Scale RNA Production Systems kits (Promega, Madison, WI, USA). The formation of the dsRNA was quantified by UV spectrophotometry and analyzed by agarose gel electrophoresis. The study was performed by injecting shrimp intramuscularly as follows: (**1**) saline control group (SS, $n = 10$), (**2**) WSSV group (100 μL WSSV inoculum, $n = 30$ total, $n = 10$/time point), and (**3**) WSSV + dsRNA group (100 μL inoculum + 15 μg of dsRNA HIF-1α simultaneously, $n = 30$ total, $n = 10$/time point). The dsRNA injection was effective for silencing HIF-1α as previously demonstrated for this experimental group (Hernández-Palomares et al., 2018).

2.2. Sample collection and preparation

Hepatopancreas and muscle ($n = 7$) were collected from the 3 groups as follows: **A**) SS group at 24 h after starting the experiment, **B**) WSSV group at 24 and 48 h post-infection, and **C**) WSSV/dsRNA group at 24, 48, and 72 h post-treatment. Our initial intent was to collect tissues from the WSSV group at 72 h, but this group experienced 100% mortality by that time point. An aliquot of each tissue was separated for the individual isolation of genomic DNA (gDNA, using the PureLink® Genomic DNA Mini Kit, Invitrogen, Carlsbad, CA, USA), total RNA (tRNA, using the TRIzol reagent, Invitrogen, Carlsbad, CA, USA), and cytoplasmic protein (using the NE-PER protein extraction kit, Pierce, Rockford, IL, USA). The remaining tissues were immediately frozen in liquid nitrogen and kept at −80 °C for later analyses.

The gDNA and tRNA concentrations and integrities were analyzed by measuring the absorbance at 260/280 nm and by 1% agarose gel electrophoresis. Contamination of genomic DNA in tRNA was eliminated by digestion with RQ1 RNase-Free DNase (Promega). Total protein concentration was measured using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA, USA). All samples were run in triplicate.

2.3. Absolute quantification of the number of copies of WSSV genomic DNA

The quantification of WSSV was done by quantitative PCR (qPCR) using specific primers for the WSSV VP-28 major envelope gene. qPCR reactions for each tissue sample were run by duplicate in the Step One Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) in a final volume of 15 μL containing 7.5 μL of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), 5.1 μL of H_2O, 0.2 μL of each primer (20 μM), and 50 ng of gDNA. After an initial denaturing step at 94 °C for 10 min, amplifications were performed for 40 cycles at 94 °C for 15 s and 63 °C for 1 min with a single fluorescence measurement and a final melting curve program increasing 0.3 °C each 20 s from 60 to 95 °C. Standard curves from serial dilutions ($1.8E^{-3}$ to $1.8E^{-7}$ ng μL^{-1}) of VP-28 PCR fragments were generated and used to calculate the number of WSSV copies using the formulations as described previously (Mendoza-Cano and Sanchez-Paz, 2013).

2.4. Quantification of antioxidant genes

Total DNA-free RNA (2.5 μg) was used to synthesize one cDNA for each sample using oligo-dT and the GoScript™ Reverse Transcriptase kit (Promega). Specific primers for each gene were designed based on the nucleotide sequences deposited in GenBank database (Table 1). The qPCR reactions were performed separately for each gene and normalized by the expression of the ribosomal protein L8. Two PCR reactions for each cDNA (2 data/sample and 14 data/group) were run (Step One Real-Time PCR Systems, Applied Biosystems) in a final volume of 15 μL containing 7.5 μL of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), 5 μL of H_2O, 0.25 μL of each primer (20 μM), and 1 μL of cDNA (equivalent to 125 ng of the original tRNA). After an initial denaturing step at 94 °C for 10 min, amplifications were performed for 40 cycles at 94 °C for 15 s and 63 °C for 1 min with a single fluorescence measurement, and a final melting curve program increasing of 0.3 °C each 20 s from 60 to 95 °C. Positive and negative controls were included for each gene. Standard curves for each gene and ribosomal protein L8 were run to determine the efficiency of amplification using dilutions from $5E^{-3}$ to $5E^{-8}$ ng μL^{-1} of PCR fragments.

2.5. Biochemical analysis

2.5.1. Superoxide dismutase (E.C. 1.15.1.1)

Total SOD activity was measured at 25 °C by mixing 590 μL of the work solution [50 mM sodium carbonate, 0.1 mM xanthine, 0.025 mM of nitro blue tetrazolium (NBT), 0.1 mM of EDTA], 1 μL of 0.1 mM xanthine oxidase and 10 μL of cytoplasmic protein extract or blank (potassium phosphate buffer). All measurements were performed in triplicate measuring the absorbance of each sample at 560 nm in a Jenway 6705 UV–vis spectrophotometer for a total of 5 min at 25 °C.

Table 1
Primers used for the qPCR of each gene.

Primer name	Nucleotide sequences (5′-3′)	GeneBank accession number	Product size (base pairs)
CatFw	GCGTGACAAGTGTGCAGAGC	AY518322.1	190
CatRv	GCTCCCTTAGCATGCACAACC		
GSTFw	CACGGGCACTGAGTTCGAGG	AY573381.2	208
GSTRv	CGTGCATGTCTGTCAGTTG		
MnSODFw	GGAAGCTTACATCTCCATCC	DQ005531.1	227
MnSODRv	GCATGAGCACCTCGTTCCTC		
L8F	GTCTACTGCGGCAAGAAGGC	DQ316258.1	197
L8R	CCTGAAGGGAGCTTTACACG		