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Hypoxia inducible factor −1 regulates WSSV-induced glycolytic genes in the white shrimp *Litopenaeus vannamei*

José Arquimídes Godoy-Lugo[a], Melissa M. Miranda-Cruz[b], Jesús Alfredo Rosas-Rodríguez[b], Norma Patricia Adan-Bante[b], Ramona Icedo-García[b], José Guadalupe Soñanez-Organis[b,*]

[a] School of Natural Sciences, University of California Merced, 5200 N Lake Road, Merced, CA, 95343, USA
[b] Universidad de Sonora, Departamento de Ciencias Químico-Biológicas y Agropecuarias, Universidad de Sonora Unidad Regional Sur, Apartado Postal 85880, Navojoa, Sonora, Mexico

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ABSTRACT

Hypoxia-inducible factor −1 (HIF-1) is a transcriptional factor that regulates the expression of several glycolytic genes. The white spot syndrome virus (WSSV) induces a shift in glycolysis that favors viral replication in white shrimp *Litopenaeus vannamei*. HIF-1 is related to the pathogenesis of the WSSV infection through the induction of metabolic changes in infected white shrimp. Although the WSSV infection is associated with metabolic changes, the role of HIF-1 on key glycolytic genes during the WSSV infection has not been examined. In this work, we evaluated the effect of HIF-1α silencing on expression and activity of glycolytic enzymes (Hexokinase-HK, phosphofructokinase-PFK and pyruvate kinase-PK) along with the glucose transporter 1 (Glut1), regulatory enzymes (glucose-6-phosphate dehydrogenase-G6PDH and pyruvate dehydrogenase-PDH), and metabolic intermediates of glycolysis (glucose-6-phosphate-G6P and pyruvate). The expression of Glut1 increased in each tissue evaluated after WSSV infection, while HK, PFK and PK gene expression and enzyme activities increased in a tissue-specific manner. G6PDH activity increased during WSSV infection, and its substrate G6P decreased, while PDH activity decreased and its substrate pyruvate increased. Silencing of HIF-1α blocked the WSSV-induced Glut1 and glycolytic genes upregulation and enzyme activity in a tissue-specific manner. We conclude that HIF-1 regulates the WSSV-induced glycolysis through induction of glycolytic genes contributing to glucose metabolism in tissues of infected shrimp. Also, the inhibition, and activation of regulatory genes are likely to decrease the availability of the raw materials essential for WSSV replication and increase oxidative metabolism.

1. Introduction

Hypoxia-inducible factor −1 (HIF-1) is a transcriptional factor formed by a regulatory α-subunit (HIF-1α) and a constitutive β-subunit (HIF-1β), and it regulates glucose metabolism in white shrimp *Litopenaes vannamei* [1]. HIF-1α is induced during hypoxia [2] and its silencing demonstrated that it regulates glycolysis through the induction of key glycolytic genes (hexokinase-HK, phosphofructokinase-PFK, fructose 1,6-bisphosphatase-FBP, Lactate dehydrogenase-LDH) [3–5].

The White Spot Syndrome Virus (WSSV) (genus *Whispovirus*, family *Nimaviridae*) is a dsDNA virus that is extremely contagious and lethal for shrimp. The WSSV can cause 100% accumulated mortality from 2 to 10 days on shrimp farms, which makes it dangerous for the wild shrimp population [6,7]. WSSV infection increases energy demand [8] and induces the Warburg effect, a shift in glycolysis in which glucose consumption and lactate production increase even in the presence of oxygen, and that is essential for successful viral replication [9,10]. These shifts in glucose metabolism increase glucose transport by the glucose transport 1 (Glut1) in WSSV-infected shrimp [11], and differentially regulates the activity of HK, PFK and PK [9,12]. Also, the WSSV infection increases the activity of the glucose-6-phosphate dehydrogenase (G6PDH) [9], which incorporates glucose-6-phosphate (G6P) into the pentose phosphate pathway (PPP), and decreases pyruvate dehydrogenase (PDH) activity [12], which incorporates pyruvate into the tricarboxylic acid (TAC) cycle.

Recently, we demonstrated that HIF-1 regulates the expression of LDH subunits during WSSV infection contributing to substrate metabolism in energetically active tissues in shrimp [10], and that HIF-1α silencing decreased the WSSV viral load along with shrimp mortality [13]. Overall, WSSV infection affects the shrimp by triggering pathways

* Corresponding author. Departamento de Ciencias Químico Biológicas y Agropecuarias Universidad de Sonora Unidad Regional Sur, Apartado Postal 85880, Navojoa, Sonora, Mexico.
E-mail address: jose.organis@unison.mx (J.G. Soñanez-Organis).

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that mimic the Warburg's effect, directly modifying normal glucose metabolism. However, the expression and activity of key glycolytic genes and enzymes during silencing of HIF-1α has not been studied in tissues from WSSV-infected shrimp. In this work, we report the changes that the WSSV infection induces to the expression and activity of key glycolytic genes (HK, PFK, and PK) and the impact of HIF-1α-silencing on these targets, along with key regulatory enzymes (G6PDH and PDH) and metabolic intermediates of glycolysis (G6P and pyruvate).

2. Material and methods

2.1. Animal handling

Juvenile *L. vannamei* shrimp (15 ± 2 g) were provided by a shrimp farm located in the state of Sonora, Mexico. Seventy healthy and specific-pathogen-free shrimp were acclimated for four weeks to laboratory conditions, being kept in aquaria of 200-L with controlled temperature (between 28 °C and 30 °C), constant aeration to maintain the dissolved oxygen (6.02 mg/L), and salinity (35 ppt). Shrimp were fed to 3% of their biomass with commercial feed pellet containing 35% protein (Purina). The water quality was maintained by constant recirculation of water through biological filters of 500 μm and UV radiation.

2.2. Silencing of HIF-1α and WSSV infection

The experimental inoculum of WSSV and the double-stranded RNA (dsRNA) were prepared as previously described [10,13]. Each experimental group was injected intramuscularly as follows: **1)** saline solution as control (SS group, n = 10) and represents baseline levels, **2)** 100 μL of WSSV inoculum (1.4×10^5 copy number) (WSSV group, n = 30), and **3)** 100 μL of WSSV inoculum (1.4×10^5 copy number) and 15 μg of dsRNA HIF-1α (WSSV/dsRNA group, n = 30). Hepatopancreas and muscle were collected as follows: SS group, at 24 h post-starting the experiment; WSSV group, at 24 and 48 h post-infection; and WSSV/dsRNA group, at 24 and 48 h post-treatment.

2.3. Quantification of glycolytic genes

Total RNA was isolated from muscle and hepatopancreas using the TRIzol reagent (Invitrogen), and genomic DNA was eliminated by digestion with DNase I (Promega). First-strand cDNAs were reverse-transcribed from DNA-free RNA (2.5 μg) using the GoScript™ Reverse Transcriptase kit (Promega) and oligo-dT. qPCR reactions were performed for Glut1, HK, PFK, and PK, and normalized by the mRNA expression of the ribosomal protein L8. Primer sequences used are shown in Table 1. Three PCR reactions for each cDNA were run (3 data for each sample and 15 data each group) using a Step One Real-Time PCR Systems (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 μL of H_2O, 0.25 μL of each primer (20 μM), and 1 μL of cDNA (equivalent to 125 ng of the original total RNA). 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. Positive and negative controls were included for each gene. Standard curves for each gene and ribosomal protein L8 were run to quantify the fluorescence and determine the efficiency of amplification using dilutions from $5E^{-4}$ to $5E^{-8}$ ng μL−1 of PCR fragments.

Table 1
Primers used for the quantitative PCR of each gene.

Primer name	Nucleotide sequences (5′-3′)	GeneBank accession number	Product size (base pairs)
Glycolytic gene			
LvGlut1F1	GCACGAAGAGCCCTAAGG	KJ701599.1	205
LvGlut1R1	GCACCGCATTAATGCCTG		
LvHKF1	GGACATAGAATGGGGTGCC	EF102106.1	221
LvHKR1	CCTGCGTATCTAACTTGCCG		
LvPFKF1	CACTCCTCCAGCTGCTGC	EF102107.1	197
LvPFKR1	GCAATGGCATCAACAGCCTC		
LvPKF1	CGGTCTGTAGAGATGCTGG	EF102105.1	202
LvPKR1	GGCCAGTACGAATTTCAGG		
L8F	GTCTACTGCGGCAAGAAGGC	DQ316258.1	197
L8R	CCTGAAGGGAGCTTTACACG		

2.4. Enzymatic activity

HK (EC 2.7.1.1) activity was measured as described previously [4]. PFK (EC 2.7.1.11), G6PDH (EC 1.1.1.49), PK (EC 2.7.1.40) and PDH (EC 2.1.4.1) activities were measured using the Phosphofructokinase Assay Kit (SIGMA-ALDRICH, MAK093), Glucose 6 Phosphate Dehydrogenase Assay Kit (Abcam, ab102529), Pyruvate Kinase Assay Kit (SIGMA-ALDRICH, MAK072) and Pyruvate Dehydrogenase Assay Kit (SIGMA-ALDRICH, MAK183) following the manufactures instructions. All measurements were performed by triplicate, measuring the absorbance of each sample at 340 (HK), 450 (PFK, G6PDH, and PDH) and 570 nm (PK) in a Varioskan LUX multimode microplate reader (Thermo Fisher Scientific). Specific activity was expressed in U μg of protein^{-1} and was defined for HK, PFK, and PDH as the amount of enzyme that will generate 1.0 μmole of NADH per minute at pH 7.5 at 37 °C, while for PK and G6PDH as the amount of enzyme that synthesizes 1 μmol of pyruvate and 6-phosphogluconolactone min^{-1} at 25 °C and 37 °C, respectively.

2.5. Glucose-6-phosphate and pyruvate

G6P and pyruvate concentrations were measured using the Glucose-6-Phosphate Assay Kit (SIGMA-ALDRICH, MAK014) and Pyruvate Assay Kit (Sigma-Aldrich, MAK071), respectively, following the manufacturer instructions. All measurements were performed in triplicate, measuring the absorbance of each sample at 450 nm and 570 nm for G6P and pyruvate, respectively, in a Varioskan LUX multimode microplate reader (Thermo Fisher Scientific). Results are expressed as nmol μL^{-1} of G6P or pyruvate μg protein^{-1}.

2.6. Statistics

Data were tested for normality and homogeneity of variance, while differences for each group were detected by one-way ANOVA with Bonferroni *post hoc* test using the saline solution-injected shrimps as control. Means (± s.e.m.) were considered statistically different at $P < 0.05$ (STATISCAL 8 software, StatSoft Inc., Tulsa, OK, USA).

3. Results

3.1. Glycolytic genes are regulated in a tissue-specific manner during WSSV infection

The expression of Glut1 increased 6–fold in hepatopancreas of WSSV group at 48 h post-infection compared to SS group, while in the WSSV/dsRNA group the expression increased 2-fold at 48 h, respectively. In muscle, Glut1 expression increased 17- and 88-fold in the WSSV group at 24 h and 48 h post-infection, respectively, compared to SS group, while in the WSSV/dsRNA group the expression increased 37- and 2-fold at 24 h and 48 h, respectively (Fig. 1A). The expression of HK remained at baseline levels in hepatopancreas of the WSSV and WSSV/dsRNA groups at each time evaluated compared to the SS group. In contrast, the expression of HK increased 35- and 141–fold in muscle of the WSSV at 24 h and 48 h post-infection, respectively, compared to the SS group, while in the WSSV/dsRNA group increased 5- and 5.5-fold at

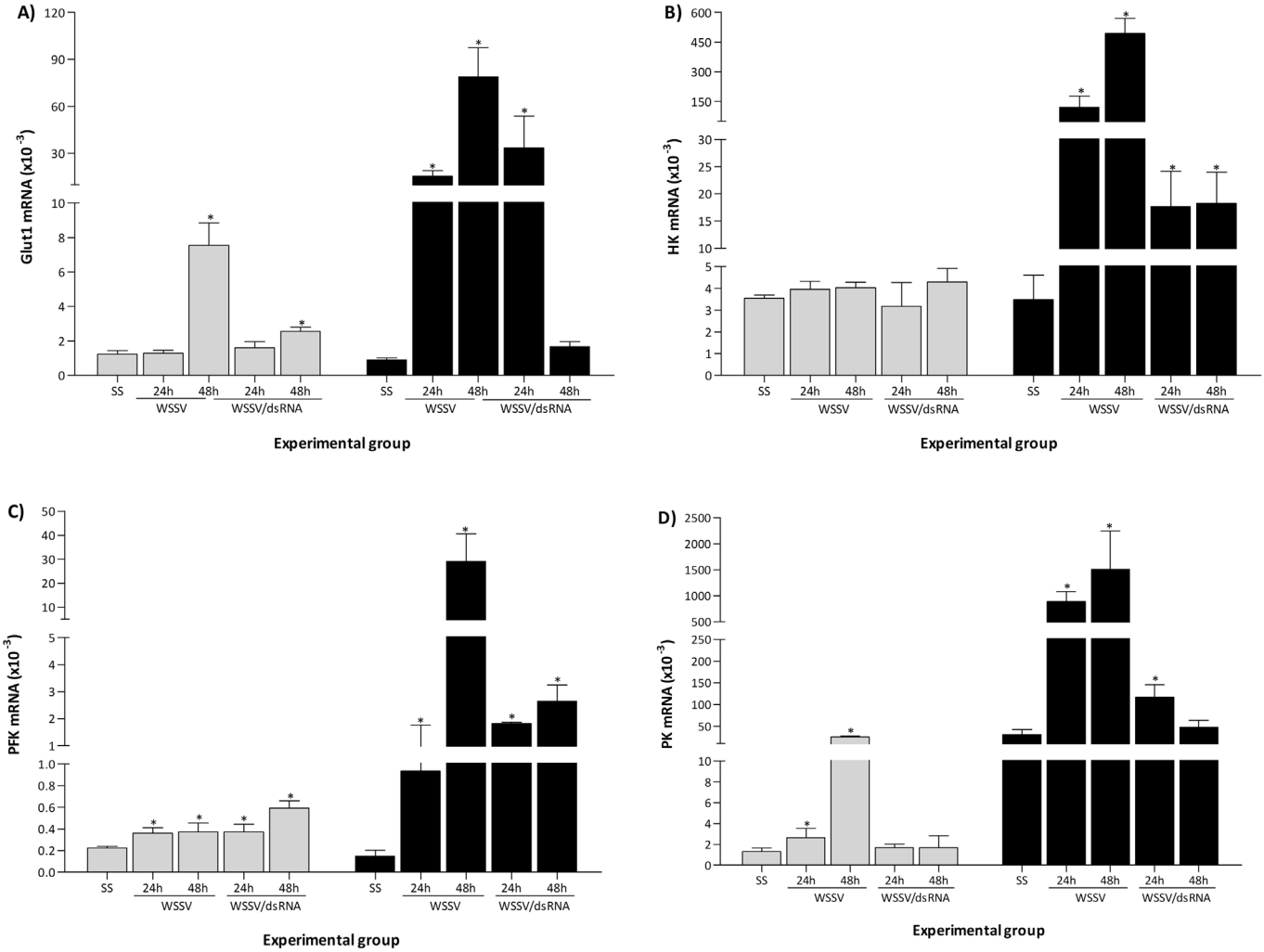

Fig. 1. Mean (± s.e.m.) Effect of HIF-1α silencing on the expression of Glut1, HK, PFK and PK in hepatopancreas (gray bars) and muscle (black bars) from WSSV-infected shrimp. The mRNA was determined by qPCR and compared to ribosomal L8 levels, n = 5. Asterisks denote significant ($P < 0.05$) differences from SS group.

24 h and 48 h, respectively (Fig. 1B). The expression of PFK increased 1.6 in hepatopancreas of the WSSV group at 24 h and 48 h post-infection, respectively, compared to the SS group, while in the WSSV/dsRNA group increased 1.6- and 3-fold at 24 h and 48 h post-treatment. In muscle, the expression of PFK increased 6.6- and 207-fold at 24 h and 48 h post-infection, respectively, compared to the SS group, while in the WSSV/dsRNA group increased 13- and 18.5-fold at 24 h and 48 h (Fig. 1C). The expression of PK increased 2- and 19-fold in hepatopancreas of WSSV group at 24 h and 48 h post-infection, respectively, compared to the SS group, while in the WSSV/dsRNA group remained at baseline levels at 24 h and 48 h post-treatment. In muscle, the expression of PK increased 30- and 50-fold at 24 h and 48 h post-infection, respectively, compared to the SS group, while in the WSSV/dsRNA increased 4-fold at 24 h post-infection, and remained at baseline levels at 48 h (Fig. 1D).

3.2. Glycolytic enzyme activities are regulated in a tissue-specific manner during WSSV infection

HK activity increased 8-fold in hepatopancreas of the WSSV group at 48 h post-infection compared to the SS group, while in the WSSV/dsRNA group the activity remained at baseline levels. In muscle, HK activity increased 1.6-fold at 48 h post-infection compared to the SS group and increased 3.6-fold in the WSSV/dsRNA at 48 h post-treatment (Fig. 2A). PFK activity increased 1.6-fold in hepatopancreas of the WSSV group at 24 h post-infection compared to the SS group, while at 48 h the activity return to at baseline levels. In the WSSV/dsRNA group, PFK activity increased 2.4-fold in hepatopancreas at 24 h post-treatment compared to the SS group, while at 48 h the activity return to at baseline levels. In muscle, PFK activity increased 3.8- and 2.6-fold in the WSSV group at 24 h and 48 h post-infection, respectively, compared to the SS group and remained increased 6.4- and 4.6-fold at 24 h and 48 h post-treatment of the WSSV/dsRNA group (Fig. 2B). PK activity increased 2.8-fold in hepatopancreas of the WSSV group at 24 h post-infection compared to the SS group, but it returned to at baseline levels at 48 h post-infection as well as at 24 h and 48 h of the WSSV/dsRNA group. In muscle, PK activity increased 3-fold at 24 h in the WSSV group and the WSSV/dsRNA group, respectively, compared to the SS group, and then returned to baseline level at 48 h in both groups (Fig. 2C).

3.3. G6PDH and PDH enzyme activities are regulated in a tissue-specific manner during WSSV infection

G6PDH activity increased 3- and 2.5- fold in hepatopancreas of WSSS group at 24 h and 48 h post-infection, respectively, compared to the SS group, while in the WSSV/dsRNA group it remained at baseline levels at 24 h and 48 h post-treatment. In muscle, G6PDH activity increased 7- and 4-fold at 24 h and 48 h post-infection, respectively, compared to the SS group, and 5- and 3-fold in the WSSV/dsRNA group at 24 h and 48 h post-treatment (Fig. 3A). PDH activity increased 1.5-

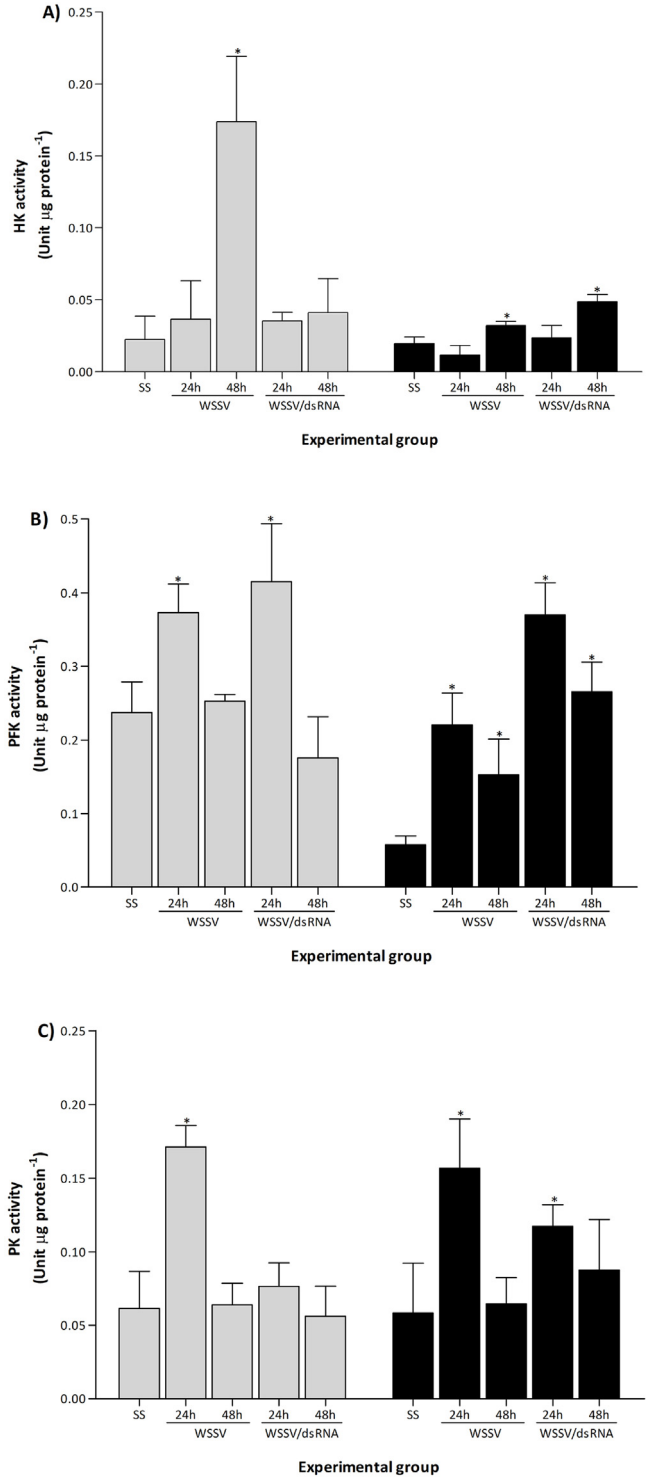

Fig. 2. Mean (± s.e.m.) Effect of HIF-1α silencing on the enzyme activity of HK, PFK and PK in hepatopancreas (gray bars) and muscle (black bars) from WSSV-infected shrimp. The asterisks denote significant ($P < 0.05$) differences from SS group. All enzyme activities are expressed in Units μg of protein^{-1}.

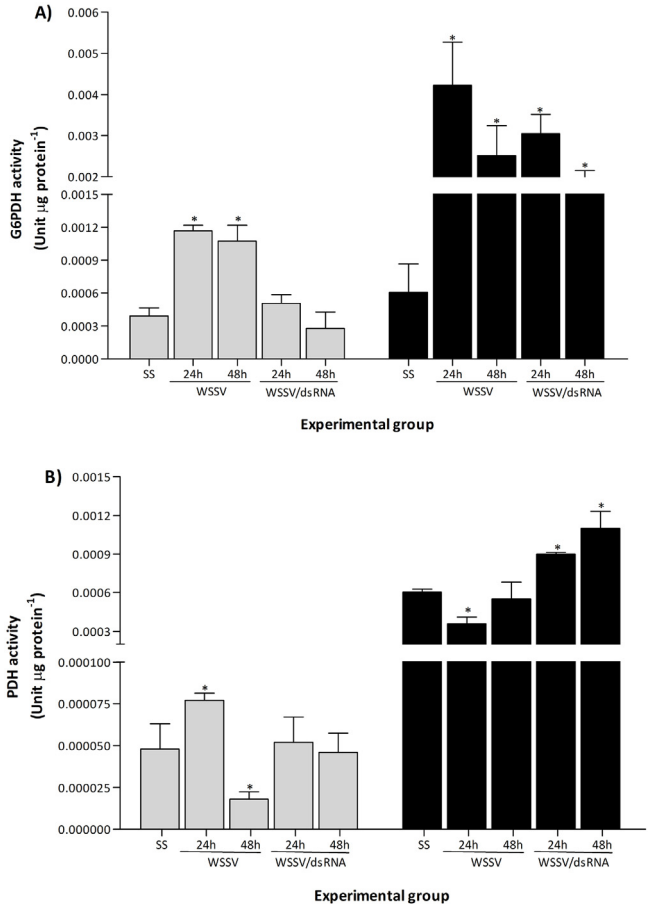

Fig. 3. Mean (± s.e.m.) Effect of HIF-1α silencing on the activity of G6PDH and PDH in hepatopancreas (gray bars) and muscle (black bars) from WSSV-infected shrimp. The asterisks denote significant ($P < 0.05$) differences from SS group. All enzyme activities are expressed in Units/μg of protein. All enzyme activities are expressed in Units μg of protein^{-1}.

fold and decreased 2.5-fold in hepatopancreas of WSSV group at 24 h and 48 h post-infection, respectively, compared to the SS group, while in WSSV/dsRNA group it remained at baseline levels at 24 h and 48 h post-treatment. In muscle, PDH activity decreased 1.7-fold at 24 h post-infection compared to the SS group, and at 48 h no changes were detected. In the WSSV/dsRNA group, PDH activity increased 1.5- and 1.8-fold at 24 h and 48 h post-treatment, respectively, compared to the SS group (Fig. 3B).

3.4. WSSV infection decreased G6P and increased pyruvate level in shrimp tissue

G6P concentration decreased 3.5- and 6-fold in hepatopancreas of WSSV group at 24 h and 48 h post-infection, respectively, compared to the SS group, while in the WSSV/dsRNA group decreased 25- and 12-fold at 24 h and 48 h post-treatment. In muscle, GP6 concentration decreased 6- and 2-fold in the WSSV group at 24 h and 48 h post-infection, respectively, compared to the SS group, and in the WSSV/dsRNA group decreased 1.5- and 3.5-fold at 24 h and 48 h post-treatment (Fig. 4A). Pyruvate concentration increased 20- and 3-fold in hepatopancreas of WSSV group at 24 h and 48 h post-infection, respectively, compared to the SS group, while in the WSSV/dsRNA decreased 6.5-fold at 24 h and 48 h. In muscle, pyruvate concentration increased 2.5- and 1.8-fold in the WSSV group at 24 h and 48 h post-infection, respectively, compared to the SS group, and in the WSSV/dsRNA group decreased 14- and 3.5-fold at 24 h and 48 h post-treatment (Fig. 4B).

4. Discussion

In the present study, we demonstrated that WSSV infection increased the mRNA expression of Glut1 in a tissue-specific manner, along with key glycolytic genes (HK, PFK, and PK) that translated into increases into their respective enzymatic activity. Furthermore, we

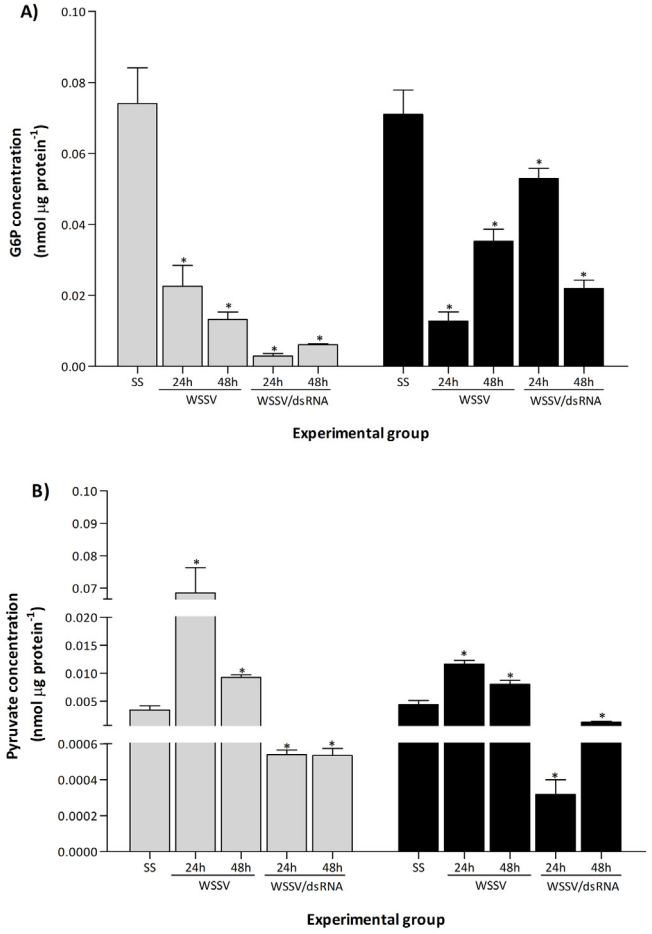

Fig. 4. Mean (± s.e.m.) Effect of HIF-1α silencing on the G6P (A) and Pyruvate (B) content in hepatopancreas (gray bars) and muscle (black bars) from WSSV-infected shrimp. The asterisks denote significant ($P < 0.05$) differences from SS group.

demonstrated that G6PDH activity increased during WSSV infection along with pyruvate concentration, while PDH activity decreased along with G6P substrate levels. Finally, silencing of HIF-1α demonstrated that the WSSV-induced Glut1 and glycolytic gene expression is regulated in a tissue-specific manner via HIF-1. We also demonstrated that the silencing of HIF-1 decreased G6PDH activity and increased PDH activity in infected shrimp.

The expression of genes involved in glucose metabolism is up-regulated during mammalian viral infections, e.g. dengue virus (Glut1 and HK2) [14], Rous Sarcoma Virus (fructose-2,6-bisphosphatase) [15], alphavirus Mayaro (PFK) [16], hepatitis B virus (Glut1) [17], hepatitis C virus (HK2) [18], Kaposi's Sarcoma-associated Herpesvirus (Glut3 and HK2) [19] and human cytomegalovirus (Glut4) [20]. In white shrimp, it has only been shown that Glut1 transports glucose during WSSV infection, and that Glut1 can also interact with the WSSV infectome [11], while expression of HK and PK is reported to be down-regulated in hemocyte and hepatopancreas during WSSV [9] and decapod *penstyldensovirus* infections [21], respectively. Also, WSSV infection up-regulates the expression of triose phosphate isomerase and enolase in cephalothorax from shrimp *Exopalamon carincauda*, and down-regulate the expression of PDH, citrate synthase and isocitrate dehydrogenase [22]. The tissue-specific expression of Glut1 and glycolytic genes may be related to the tissue function and presence of other Glut isoforms. The hepatopancreas participates in the absorption and storage of nutrients, and carbohydrate and lipid metabolism; the muscle has high energy requirements and glucose intake for its locomotor function. In mammals, the genes that encode for the different isoforms of Glut1 [23], HK (Hex-A, -B, –C and D) [24–26], PFK [27] and PK [28–30] are expressed in a tissue-specific manner during different stress conditions. In white shrimp, two Glut (1 and 2) isoforms have been identified [31,32], but isoforms for HK, PFK and PK have not been reported yet. Therefore, the observed increases of tissue-specific mRNA expression of Glut1 and glycolytic genes suggest the existence of isoforms for these enzymes and demonstrates that it contributes to the correct glucose supply and induction of glycolysis during the WSSV infection.

The increased flow of glycolysis has been shown in hemocytes [9,33] and tissue [10] of WSSV-infected shrimps. The activity of HK, which catalyze the conversion of glucose to G6P, decreases after 24 h post-infection with the WSSV infection [9]. Meanwhile, the activity of PFK, which catalyzes the phosphorylation of fructose 6-phosphate (F6P), increases from 1 to 24 h post-infection with WSSV [12]. The activity of PK, which catalyzes the conversion of phosphoenolpyruvate (PEP) to pyruvate, is inhibited in WSS-infected shrimp after 6, 12 and 24 post-infection [12]. Also, WSSV infection increases the protein level of several glycolytic enzymes (HK, aldolase, enolase, and PK) in hemocyte of infected white the shrimp [33]. Our results demonstrate that the WSSV infection increases the activity of HK, PFK, and PK in energetically active tissues of infected shrimp suggesting an increase in the flow of glycolysis and the concentration of glycolytic intermediates, which satisfy the energic demand and availability of the raw materials essential for WSSV replication. The lack of a correlation between the abundance of mRNA and enzymatic activity in white shrimp has been shown for HK [4], PFK [3] and LDH [5], and which can also a consequence of post-transcriptional and post-translational regulation, as it is the case for mammals [34–37]. Also, different HK (I-IV) [38], PFK (M, L and P) [39] and PK (M, L and R) [28] isoenzymes are known in mammals, and they differ in its catalytic activity and roles in specific metabolic status, further supporting the suggestion of the presence of isoforms that drive the glycolytic response during WSSV viral infection.

G6P and pyruvate are intermediates of glycolysis used as sources for PPP and TCA cycle. During viral infections, PPP provides free nucleotide pools for rapid viral genome replication [40–44], while the TCA cycle is associated to lipid biosynthesis necessary for successful viral envelope formation [45,46] and energy generation [47–49]. The WSSV infection increases G6PDH activity in hepatopancreas and muscle of tiger prawns *Penaeus monodon* at 24 h post-infection [50], and hemocyte of white shrimp at 12 h, 36 h, and 72 h post-infection [9]. In contrast, the activity of PDH is decreased in hemocyte [12] and cephalothorax [33] of WSSV infected shrimp. On the other hand, WSSV increases the content of glycolytic metabolites (glucose, G6P, dihydroxyacetone phosphate, glyceraldehyde 3-phosphate, 2-phosphoglycerate, F6P, PEP, F1,6BP and pyruvate), and decrease the content of TCA cycle metabolites (Acetyl-CoA and alanine) in hemocytes of WSSV infected shrimps [33]. Also, the concentration of TCA cycle metabolites (fumarate, succinate, malate, and alpha-ketoglutarate) is decreased in hepatopancreas of WSSV infected white shrimp [51]. Our results agree with the previously cited literature, supporting the idea that the activation of the PPP and partial disruption of the TCA cycle happens during the WSSV infection in white shrimp. Also, the increase of the activity of PDH in hepatopancreas at 24 h post-infection suggests an activation of the TCA cycle to satisfy the energetic demand of the WSSV to then be shut down by pyruvate deprivation produced by the takeover of aerobic glycolysis, present during the WSSV-induced Warburg's effect [9].

In mammalian, certain viral infections induce aerobic glycolysis (also called Warburg effect) for successful viral replication and virion production [44,52–55], and in which an increase in glucose consumption and lactate production, even in the presence of oxygen, is seen [56]. The Phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) (PI3K/Akt/mTOR) [57–62] and HIF-1 [40,55,63,64] are signaling pathways used by viruses to regulate these changes, and they are reported to be required for

effective virus replication. In contrast, few studies describe the molecular mechanisms of metabolic regulation in WSSV-infected shrimp. Su et al., 2014 demonstrated that the WSSV induces a shift in glycolysis via the PIK3-Akt-mTOR pathway for successful viral replication in hemocytes of infected white shrimp [33]. We have shown previously that the WSSV infection induces the expression of HIF-1 in tissues from white shrimp. Additionally, silencing of HIF-1α blocked the glucose consumption and lactate generation production produced by the WSSV infection through the regulation of the expression of LDH subunits in highly energetic tissues (gills, hepatopancreas, and muscle) [10]. Also, we have demonstrated that the silencing of HIF-1α decreases the WSSV viral load in infected shrimp along with their mortality [13] suggesting that HIF-1 contributes to the pathogenesis of WSSV through the induction of a Warburg Effect-like response [9]. Therefore, the blockade of the WSSV-induced glycolytic gene expression by the silencing of HIF-1α demonstrates that HIF-1 regulates the WSSV-induced increase of glycolysis influx, which ultimately leads to changes in the availability of metabolic intermediates used for other metabolic pathways, those being the PPP and TCA cycle.

In conclusion, this study demonstrates that HIF-1 cooperates to mediate changes in glucose metabolism by up-regulating target genes associated with glycolysis in highly energetic tissues of WSSV-infected white shrimp. Moreover, G6PDH activity was decreased during the silencing of HIF-1 while PDH increased, suggesting that the raw materials essential for WSSV replication would scarce and that activation of PDH increase oxidative metabolism.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

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