White Spot Syndrome Virus Induces Metabolic Changes Resembling the Warburg Effect in Shrimp Hemocytes in the Early Stage of Infection[▽]

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The Warburg effect is an abnormal glycolysis response that is associated with cancer cells. Here we present evidence that metabolic changes resembling the Warburg effect are induced by a nonmammalian virus. When shrimp were infected with white spot syndrome virus (WSSV), changes were induced in several metabolic pathways related to the mitochondria. At the viral genome replication stage (12 h postinfection [hpi]), glucose consumption and plasma lactate concentration were both increased in WSSV-infected shrimp, and the key enzyme of the pentose phosphate pathway, glucose-6-phosphate dehydrogenase (G6PDH), showed increased activity. We also found that at 12 hpi there was no alteration in the ADP/ATP ratio and that oxidative stress was lower than that in uninfected controls. All of these results are characteristic of the Warburg effect as it is present in mammals. There was also a significant decrease in triglyceride concentration starting at 12 hpi. At the late stage of the infection cycle (24 hpi), hemocytes of WSSV-infected shrimp showed several changes associated with cell death. These included the induction of mitochondrial membrane permeabilization (MMP), increased oxidative stress, decreased glucose consumption, and disrupted energy production. A previous study showed that WSSV infection led to upregulation of the voltage-dependent anion channel (VDAC), which is known to be involved in both the Warburg effect and MMP. Here we show that double-stranded RNA (dsRNA) silencing of the VDAC reduces WSSV-induced mortality and virion copy number. For these results, we hypothesize a model depicting the metabolic changes in host cells at the early and late stages of WSSV infection.

During the early stage of most lytic viral infections, when the virus genome is being replicated, viral products will modulate host cell metabolic homeostasis to boost activities such as glycolysis, the pentose phosphate pathway (PPP), and fatty acid metabolism in order to favor pathogen biosynthesis and fulfill the pathogen's energy requirements. Subsequently, at the late stage of infection, when virion maturation is complete, damage to the cell's metabolism can lead to cell death, which in turn allows the new virions to be released. Using cell culture systems and state-of-the-art techniques, systems-level metabolic flux profilings have monitored these events in several mammalian viruses, including human cytomegalovirus (HCMV) and hepatitis C virus (HCV) (6, 20, 21). In some viruses, such as HCV and Kaposi's sarcoma herpesvirus (KSHV), these metabolic changes are characteristic of the Warburg effect (5, 6). The Warburg effect, which is an abnormal glycolytic response that is associated with cancer cells and tumors, involves the mitochondria and is partly mediated by the voltage-dependent anion channel (VDAC) (19). Interestingly, the VDAC also

plays a critical role in cell death via its involvement in mitochondrial membrane permeabilization (MMP) (27, 30).

In a global study of the changes in protein expression levels in the stomachs of shrimp infected by the large double-stranded DNA (dsDNA) shrimp virus white spot syndrome virus (WSSV) (genus *Whispovirus*, family *Nimaviridae*), many of the upregulated proteins were found to be related to mitochondrial functions (31, 36). A follow-up study focused on the role of the VDAC during WSSV infection and found that when the VDAC was silenced, infection was delayed (37). Since the VDAC is the most abundant protein in the outer mitochondrial membrane and serves as a component of the mitochondrial permeability transition pore (PTP), we hypothesized that the VDAC is probably being used by the virus to regulate host mitochondrial functions during WSSV pathogenesis. In the first part of the present study, we further investigated this hypothesis, first by observing mortality and virus copy number in WSSV-challenged shrimp after VDAC silencing and then by looking at WSSV's ability to trigger cell death by MMP, which is a process mediated by the PTP.

As noted above, there is also a connection between the VDAC and the Warburg effect. The Warburg effect is characterized by high glycolysis efficiency and increased lactate fermentation even under aerobic conditions (19), and the mitochondria are closely involved in these changes. In cancer cells,

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the VDAC and its interaction on the outer mitochondrial membrane with the glycolytic enzyme hexokinase (HK) are thought to be a key component of the underlying mechanism (19). To date, the VDAC has not been reported to be involved in any of the virally induced instances of the Warburg effect, but given its involvement in cancer cells and its upregulation in WSSV-infected shrimp, it seems a likely mechanism. In the second part of this study, we therefore investigated the possibility that the Warburg effect is being induced in cells infected with WSSV. To do this, we monitored the changes induced by WSSV in several metabolic pathways that are related to the mitochondria. This is the first study to explore whether the Warburg effect might occur *in vivo* during infection by a non-mammalian virus.

MATERIALS AND METHODS

Experimental animals and virus inoculum. For the dsRNA silencing experiment, adult kuruma shrimp (*Marsupenaeus japonicus*, also named *Penaeus japonicus*; mean weight, 10 g) were purchased from a commercial culture farm in Japan (Yamaguchi, Japan). For the shrimp cell experiments, *Litopenaeus vannamei* (also named *Penaeus vannamei*) shrimp were purchased from a commercial farm in Pingtung, Taiwan. After arriving in the respective labs, both sets of experimental shrimp were cultured in water tank systems containing filtered seawater (30 ppt at 25 to 27°C) for 1 week before the experiments. The virus used throughout this study was white spot syndrome virus (WSSV) (TW-1 isolate). The virus stock for the experimental inoculum was prepared from the hemolymph of WSSV-infected moribund shrimp, diluted with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄), and kept at -80°C. The stock was further diluted with PBS before use as the experimental inoculum. Since the susceptibility to WSSV is different for the two shrimp species used in these experiments, a different challenge titer was used for each species. These titers were adjusted such that the cumulative mortality reached approximately 50% at 3 days after WSSV challenge and 100% at 5 days after challenge. Once these titers had been determined, they were used consistently for the respective species throughout this study.

Knockdown of MjVDAC expression by dsRNA-mediated RNA interference. Preparation and injection of the dsRNA were done as described previously (18, 37). Briefly, the partial DNA fragments of the VDAC in *Marsupenaeus japonicus* (MjVDAC) and enhanced green fluorescent protein (EGFP) were generated by PCR and used as linearizing DNA templates for single-stranded RNA (ssRNA) synthesis using the T7 RiboMAX Express large-scale RNA system (Promega). After being verified by agarose gel electrophoresis and quantified by UV spectrophotometry, dsRNAs were stored at -80°C. The experimental group was challenged by intramuscular injection with MjVDAC dsRNA (1 µg/g shrimp in 100 µl PBS), while the control groups were injected with EGFP dsRNA or PBS only.

RT-PCR. Reverse transcription-PCR (RT-PCR) was used to monitor the RNA interference efficiency. Total stomach RNAs were extracted by using TRIzol reagent (Invitrogen) on samples taken from different groups (receiving MjVDAC dsRNA, EGFP dsRNA, or PBS injection) on various days postinjection (dpi) (0.25, 1, 3, and 7 dpi). Superscriptase II reverse transcriptase (Invitrogen) was used to synthesize cDNA, and anchor-dTV (5'-GACCACGCGTATCGATGTCGACT₁₆V-3') was used as the anchor primer. Reaction cycles were as follows: 95°C for 5 min, followed by 25 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min, with one final elongation stage at 72°C for 10 min and 16°C for 20 min. Elongation factor 1 alpha (EF1-α) and the inner mitochondrial membrane protein adenine nucleotide translocase (MjANT) were used as internal controls. The reverse transcription-PCRs were performed using the gene-specific primer sets MjVDAC-F/MjVDAC-R (5'-CTCAACACAGAGGTTGC C-3' and 5'-AGAGCCAGCAGCCCATGA-3'), EF1-α-F/EF1-α-R (5'-ATGGT TGTCAACTTTGCCC-3' and 5'-TTGACCTCCTTGATCACACC-3'), and MjANT-F/MjANT-R (5'-CCCATATGTCTAAGGGTTTCGATC-3' and 5'-CC CTCGAGTTATTCGCCACTCTTCGA-3').

Cumulative mortality after WSSV challenge in MjVDAC-knockdown shrimp. Shrimp (*M. japonicus*) were randomly divided into 3 groups (*n* = 40 in each group) and pretreated with MjVDAC dsRNA, EGFP dsRNA, or PBS by intramuscular injection. dsRNA silencing has been shown to become effective within 3 days after injection (37), so at 3 days after pretreatment, all of the shrimp were

challenged with WSSV. After challenge, the cumulative mortality was recorded each day.

Differences in mortality between the experimental groups were tested for statistical significance using the Kaplan-Meier plot (log rank χ^2 test) (38). The statistical procedures were carried out using MedCalc statistical software version 11.2 (Mariakerke, Belgium).

Determination of WSSV genome copy number in MjVDAC-knockdown shrimp. For this experiment, shrimp (*M. japonicus*) were randomly divided into three groups and injected with MjVDAC dsRNA, EGFP dsRNA, or PBS. At 3 days after dsRNA injection, shrimp were challenged with WSSV. Three samples were collected from the surviving shrimp in each group at various time points (1, 3, 4, and 6 days postchallenge). Their swimming legs were removed and subjected to genomic DNA extraction using a dodecyltrimethylammonium bromide (DTAB)/cetyltrimethylammonium bromide (CTAB) DNA extraction kit (Farming IntellGene Tech, Taiwan).

For real-time quantitative PCR (qPCR), specific primers were designed based on the EF1-α housekeeping gene (EF1-α-qF/EF1-α-qR, 5'-ACGTGTCCGTG AAGGATCTGAA-3' and 5'-TCCTTGGCAGGGTCGTTCTT-3') and the WSSV vp28 major envelope gene (vp28-qF/vp28-qR, 5'-AGTTGGCACCTTTG TGTGTGGTA-3' and 5'-TTTCCACCGCGGTAGCT-3'). Using an ABI PRISM 7000 sequence detection system with Brilliant SYBR green QPCR master mix (Applied Biosystems), qPCR was performed and the relative number of copies of WSSV genomic DNA was calculated as follows. First, the standard curves were generated from serial dilutions (10^6 -, 10^5 -, 10^4 -, 10^3 -, 10^2 -, and 10^1 -fold) of plasmids containing fragments of the vp28 and EF1-α genes. The genomic DNA copy numbers were then calculated by reference to these two standard dilution curves and expressed in relative units.

Determination of mitochondrial membrane potential using JC-1 staining in primary cultures of shrimp hemocytes collected from PBS- and WSSV-injected shrimp. A primary shrimp hemocyte culture system was prepared as described previously with some modifications (4, 39). Briefly, for analysis of mitochondrial membrane potential after WSSV injection, shrimp (*L. vannamei*) were randomly divided into two groups. The experimental group was challenged with WSSV, while the control group was injected with PBS only. Hemolymph was collected from the PBS- and WSSV-injected groups at various time points using a syringe that contained cooled anticoagulant solution (2% NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid, 10 mM EDTA). Three pooled samples were collected from each group at different time points, with each pooled sample being taken from 3 shrimp. A coverglass was placed at the bottom of each well of a 24-well plate, and 200 µl of culture medium (2× Leibovitz's 15 [L-15] medium with 10% fetal bovine serum [FBS], 1% glucose, 0.005% NaCl, 100 U/ml penicillin, 100 U/ml streptomycin, and 1.25 µg/ml amphotericin B [Fungizone]) (39) was added. Each pooled hemolymph sample was seeded into 3 of the wells and allowed to incubate for 2 h at room temperature. The resulting hemocyte monolayers on the coverglasses were then washed twice in 2× L-15 medium, and the cells were stained with JC-1 dye (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) in 2× L-15 medium (5 µg/ml) for 30 min at room temperature. This was followed by counterstaining with DAPI (4',6-diamidino-2-phenylindole) for 5 min to indicate the nucleus. The cells were washed two more times, and fluorescent signals at specific excitation frequencies (485 ± 11 nm and 535 ± 17.5 nm for JC-1 and 350 nm for DAPI) were observed with a microscope. For each coverglass, areas of high cell density were located using the microscope, and results were expressed as the mean (*n* = 3) of the proportion of permeabilized cells relative to the total number of cells in the microscope field. Data were subjected to unpaired Student *t* tests. *P* values of less than 0.05 were considered to be statistically significant. As a positive control, UV light treatment (15) was applied for 1 h to some of the samples after the incubation stage.

Determination of ADP/ATP ratio and H₂O₂ concentration in shrimp hemocytes collected from PBS- and WSSV-injected shrimp. For these analyses, shrimp (*L. vannamei*) were randomly divided into two groups. The experimental group was challenged with WSSV, while the control group was injected with PBS only. For hemocyte collection, at various time points after challenge, 24 individuals were randomly selected from each of the groups, and 2× four sets of pooled samples were prepared, with 3 different individuals contributing to each pooled sample. The first four sets were used for ADP/ATP ratio analysis, and the second four sets were used to determine H₂O₂ concentration. For the ADP/ATP analysis, each pooled sample was then centrifuged at 3,000 × *g* for 10 min at 4°C to form a pellet. The ADP/ATP ratio in the hemocytes in the resulting pellet was determined using an ApoSENSOR ADP/ATP ratio assay kit (Biovision Research, Mountain View, CA) according to the manufacturer's instructions.

To determine H₂O₂ concentration, the samples were centrifuged at 9,000 × *g* for 3 min at 4°C. After rinsing with cold 1× PBS to remove the remaining hemolymph, the resulting pellets were carefully suspended with 200 µl 0.33 ×

PBS and placed on ice for 10 min. The suspensions were then centrifuged at $15,300 \times g$ for 5 min, and the supernatant (i.e., hemocyte lysate) was transferred into new tubes. An Amplex Red hydrogen peroxide/peroxidase assay kit (Invitrogen) was used to measure the amount of H_2O_2 in the lysate. The kit provided standard samples with different H_2O_2 concentrations (0, 0.625, 1.25, 2.5, 5, 10, and 20 μM), and these were used to generate the standard curve. The hemocyte lysates were diluted as necessary with $0.33 \times$ PBS to arrive at concentrations that were well inside the range of the standard curve. In a 96-well plate, hemocyte lysates or standard samples (50 μl /sample) were mixed with Amplex Red reagent-horseradish peroxidase (HRP) working solution (50 μl) and incubated for 30 min at room temperature in the dark. The absorbance of each sample was then measured at 560 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader. The protein concentration in each sample was determined using a Bradford protein assay kit (Bio-Rad).

The data were reported as means $\pm$ standard deviations (SD), and unpaired Student *t* tests were used to test for statistically significant differences ($P < 0.05$).

Determination of the concentrations of glucose, lactate, and triglyceride in shrimp hemolymph after WSSV infection. Four pooled hemolymph samples were collected from *L. vannamei* without the use of shrimp anticoagulant solution at different time points after PBS or WSSV injection, with each pooled sample being taken from 3 shrimp. The samples were centrifuged at $800 \times g$ for 30 min at $4^\circ C$, and the supernatants were transferred into new tubes. These supernatants were then assayed as described below to determine the concentrations of glucose, lactate, and triglyceride.

The glucose concentration was measured by the glucose oxidase enzymatic catalyzed method using a glucose-phenol-aminophenazone (glucose-PAP) assay kit (Randox, United Kingdom). Each sample (2 μl) was mixed with 200 μl of glucose reagent in a single well in a 96-well plate. After incubation at $25^\circ C$ for 25 min, the absorbance of the sample was measured at 500 nm with an ELISA plate reader. A standard curve was generated from a set of glucose standards ranging from 0.0 to 0.495 mg/ml.

The lactate concentration was determined by an enzymatic colorimetric method using a lactate test kit (Fortress Diagnostics Limited, United Kingdom). Lactate was first oxidized into pyruvate and then further peroxidized to form purple peroxidase products. All the procedures were protected from light. Each sample (2 μl) was mixed with 200 μl of lactate working reagent per well in a 96-well plate. After incubation at $25^\circ C$ for 10 min, the absorbance of the sample was measured at 550 nm with an ELISA plate reader. A standard curve was generated from a set of lactate standards ranging from 0.0 to 0.2 mg/ml.

The triglyceride was quantitated by the glycerol-3-phosphate oxidase-PAP (GPO-PAP) method using a triglyceride test kit (Fortress Diagnostics Limited, United Kingdom). Each sample (2 μl) was mixed with 200 μl of a triglyceride working reagent per well in a 96-well plate. After incubation at $25^\circ C$ for 10 min, the absorbance of the sample was measured at 500 nm with an ELISA plate reader. A standard curve was generated from a set of triglyceride standards ranging from 0 to 2 mg/ml.

Determination of G6PDH activity. Glucose-6-phosphate dehydrogenase (G6PDH) activity was measured using a G6PDH assay kit (BioVision Inc.). Shrimp (*L. vannamei*) hemocyte samples were collected as described above. After centrifugation at $9,000 \times g$ for 3 min, the supernatant was discarded and the pellets were rinsed with cold $1 \times$ PBS, resuspended carefully with 200 μl $0.33 \times$ PBS and placed on ice for 10 min. The suspension was centrifuged again at $15,300 \times g$ for 5 min, and the supernatant was transferred into new tubes. Using a 96-well plate system, lysate samples (50 μl) were mixed with a reaction mixture (50 μl /reaction) containing 46 μl G6PDH assay buffer, 2 μl G6PDH substrate, and 2 μl G6PDH developer. Incubation proceeded at $37^\circ C$, and after 30 min (T_1), the absorbance of each sample was measured at 450 nm with an ELISA plate reader to give the A_1 value. After a total of 50 min of incubation (T_2 ; i.e., 20 min after T_1), the absorbance of each sample was measured again at 450 nm to give the A_2 value. An NADH standard curve was generated from a series of concentrations ranging from 0 to 12.5 nmol and used to convert the difference in absorbance ($A_2 - A_1$) to the NADH amount (B) generated during the period. The G6PDH activity was then calculated as follows: G6PDH activity (mU/mg) = $B / [(T_2 - T_1) \times V \times \text{total protein}]$, where V is the volume of the sample (in ml) in each reaction mixture, and the total protein was determined using a Bradford protein assay kit (Bio-Rad).

Absolute quantification of the number of copies of WSSV genomic DNA using the IQ REAL WSSV quantitative system. To absolutely quantify the temporal change in WSSV genomic DNA copy numbers postinfection, the IQ REAL WSSV quantitative system (Farming IntelliGene Tech, Taiwan) was applied. This real-time PCR system uses a TaqMan assay strategy with a dual-color design for quantifying both viral and shrimp genomic copies within a single reaction. Four pooled pleopod samples were collected from *L. vannamei* at different time

FIG. 1. Time course of MjVDAC expression in the shrimp stomach after specific RNA knockdown mediated by dsRNA injection. At each time point after dsRNA injection, total RNA was extracted from the stomach and reverse transcribed to cDNA. Injections of EGFP dsRNA and PBS were used as dsRNA controls. The EF1- α and MjANT genes were used as an internal control and a non-dsRNA target gene control, respectively.

points after WSSV injection, with each pooled sample being taken from 3 shrimp. The total genomic DNA (i.e., both shrimp and viral genomic DNAs) was extracted from the samples using a DTAB/CTAB DNA extraction kit (Farming IntelliGene Tech, Taiwan). Real-time qPCR was performed using an ABI PRISM 7300 sequence detection system following the instructions provided in the IQ REAL WSSV manual. A standard curve was generated from serial dilutions (10^6 , 10^5 , 10^4 , 10^3 , 10^2 , and 10^1 copies/ μl) of the Dual P (+) standard provided with the kit (each μl of which contains 10^6 copies of fragments of both WSSV and shrimp genomic DNAs). The virion copy numbers were then calculated by reference to the Dual P (+) standard dilution curve.

Relative quantification of the number of copies of WSSV vp28 mRNA. The vp28 mRNA copy number was determined using real-time qPCR. For the templates, RNA was extracted from pooled samples of hemolymph taken from three WSSV-challenged *L. vannamei* shrimp, and Superscriptase II reverse transcriptase (Invitrogen) was used to synthesize cDNA with the anchor dTv primer as described above. qPCR was performed with the primer sets EF1- α -qF/EF1- α -qR and vp28-qF/vp28-qR using an ABI PRISM 7000 sequence detection system with Brilliant SYBR green QPCR master mix (Applied Biosystems). The relative copy number was calculated by the $2^{-\Delta CT}$ method as described by Wang et al. (38).

RESULTS

Suppression of the shrimp VDAC leads to decreased mortality after WSSV infection. The ability of MjVDAC dsRNA to specifically suppress MjVDAC was tested using RT-PCR. Figure 1 shows that MjVDAC mRNA expression was noticeably decreased from the first day after injection through to 7 dpi. There was no suppressive effect on the MjANT gene, which encodes a major mitochondrial inner membrane protein, or on the EF1- α housekeeping gene. Based on these and previous results (37), experimental shrimp were challenged with WSSV at 3 days after dsRNA injection in the following experiments.

In the challenge test (Fig. 2), the cumulative mortality of the PBS group reached 100% at 6 days postinjection. Cumulative

FIG. 2. Gene silencing of shrimp MjVDAC decreases mortality after WSSV injection. Cumulative mortalities of shrimp challenged with PBS (control) or WSSV were observed after injection with PBS or double-stranded RNAs corresponding to MjVDAC or EGFP. Mortality was measured in each treatment group ($n = 40$) and was recorded every 24 h postchallenge. Differences in mortality levels between the MjVDAC dsRNA group and the EGFP dsRNA group were analyzed by Kaplan-Meier log rank χ^2 tests. Significant differences in shrimp mortality are marked with asterisks and were found from 3 dpi to the end of the experiment ($P < 0.05$).

mortality in the EGFP dsRNA group was lower than that in the PBS-treated controls, presumably due to sequence-independent RNA interference (37, 38), but it was also significantly higher than that in the MjVDAC dsRNA group, starting at 3 dpi ($P < 0.005$). The final mortality of the EGFP dsRNA group (85%) was also significantly greater than that of the MjVDAC

dsRNA group (47.5%) (Kaplan-Meier log rank χ^2 , 17.8656; $P < 0.0001$). The results are consistent with our previous work, which suggested that the mitochondria might play an important role in WSSV pathogenesis (37).

Suppression of the shrimp VDAC reduces WSSV genomic DNA copy number after WSSV challenge. Figure 3 shows that the numbers of copies of WSSV genomic DNA in the shrimp pretreated with MjVDAC dsRNA were significantly lower than those in the EGFP dsRNA group or the PBS group throughout the 6 days of the experiment. (There are no data for the PBS group at day 6 because the mortality in this group was already close to 100% at this time.) These results are consistent with the mortality data (Fig. 2) and clearly suggest that the mitochondria are involved in WSSV pathogenesis.

WSSV causes mitochondrial membrane permeabilization in shrimp hemocytes. To characterize details of the mitochondrial response after WSSV infection, mitochondrial membrane permeabilization (MMP), which is a key event of mitochondrion-mediated cell death, was examined in shrimp hemocytes. One of the consequences of MMP is the loss of mitochondrial membrane potential. In this study, the hemocytes isolated from WSSV-infected and control shrimp were stained with JC-1, which forms red fluorescent J aggregates at normal mitochondrial membrane potential (Fig. 4A, column a) but exists in a green fluorescent monomeric form when the mitochondrial potential drops (Fig. 4A, column b). The first two columns in Fig. 4A confirm that the shrimp hemocytes lose mitochondrial membrane potential after *in vitro* UV exposure, which is a stress stimulator known to trigger MMP.

Our results show that WSSV injection led to a loss of mitochondrial membrane potential at 24 h postinfection (hpi) and

FIG. 3. Gene silencing of shrimp MjVDAC decreases WSSV DNA copy numbers after WSSV injection. Real-time qPCR was used to measure the number of copies of WSSV genomic DNA in WSSV-challenged shrimp that had been preinjected with PBS or double-stranded RNAs corresponding to MjVDAC or EGFP. An asterisk indicates a significant statistical difference between bracketed groups ($P < 0.05$).

FIG. 4. WSSV induces mitochondrial membrane permeabilization (MMP) in shrimp hemocytes. (A) After being injected with PBS or WSSV, shrimp hemocytes were collected at the indicated times and seeded into 24-well plates. JC-1 staining was used to indicate normal (red) versus low (green) mitochondrial membrane potential. DAPI (blue) staining was used to counterstain the nucleus. UV light treatment was used as the positive control. (B) Percentage of hemocytes showing mitochondrial membrane potential loss after WSSV infection. After seeding and staining, 300 to 1,500 hemocytes in each treatment were counted at different time points. Each bar represents the mean $\pm$ SD from 3 samples, where each sample was pooled from 3 shrimp. Asterisks indicate a significant difference between groups ($P < 0.05$).

48 hpi (Fig. 4A, columns e and f), whereas PBS injection had no significant effect (Fig. 4A, columns c and d). Figure 4B quantifies the percentages of hemocytes that lost mitochondrial membrane potential in the various groups. These results suggest that WSSV induces mitochondrial membrane permeabilization in shrimp hemocytes.

Changes in ADP/ATP ratio and H_2O_2 concentration in WSSV-infected shrimp hemocytes. Changes in the ADP/ATP ratio are a reflection of mitochondrial activity and provide an indication of the cellular energy state. Figure 5A shows that this ratio became significantly higher in the WSSV group at 24 hpi and remained high to the end of the experiment (72 hpi).

To further investigate the consequences of MMP, we monitored oxidative stress, which occurs when reactive oxygen species (ROS) are released. For this study, we measured the levels of the ROS hydrogen peroxide (H_2O_2) in shrimp hemocytes. The H_2O_2 concentration in the WSSV-challenged group was significantly lower at 12 hpi and significantly higher at 24 hpi

than that in the PBS control group, but from 36 to 60 hpi, there was no significant difference between the groups.

WSSV infection significantly affects the levels of glucose and lactate in shrimp plasma. Since the glycolytic pathway is upstream from the mitochondrial citric acid pathway, we next measured the levels of glucose and its lactate downstream metabolite in plasma samples collected from shrimp after WSSV challenge or PBS injection. As Fig. 6A shows, in the WSSV group, glucose levels were significantly lower than those in the PBS controls at 12 hpi and were significantly higher at 24 hpi. Thereafter plasma glucose levels in the WSSV group fell off considerably, and they remained low to the end of the experiment. In the first 24 h, the reverse pattern was observed for plasma lactate levels (Fig. 6B); that is, lactate was higher in the WSSV group at 12 hpi and lower at 24 hpi.

G6PDH activity in shrimp hemocytes increases at 12 h after WSSV infection. Several intermediates of glycolysis and gluconeogenesis can be used as sources for other metabolic path-

FIG. 5. WSSV causes an increase in the ADP/ATP ratio, but not H₂O₂ concentration, in shrimp hemocytes. (A) Each bar represents the mean $\pm$ SD of the ADP/ATP ratios in 4 samples of pooled hemocyte lysate. Statistical significance is indicated by single ($P < 0.05$) or double ($P < 0.005$) asterisks. (B) Each bar represents the H₂O₂ concentrations (mean $\pm$ SD) in 4 pooled samples of shrimp hemocyte lysates. ND, no data. An asterisk indicates a significant statistical difference between groups ($P < 0.05$).

ways. These include glucose-6-phosphate (G6P), which is catalyzed by the key regulatory enzyme glucose-6-phosphate dehydrogenase (G6PDH) in the first step of the pentose phosphate pathway (PPP). As Fig. 7 shows, G6PDH activity increased significantly in the WSSV group at 12 hpi and then fell back to its original levels. Activity was also significantly higher in the WSSV group at 36 hpi and 72 hpi, but no clear pattern was observed.

WSSV infection leads to the collapse of triglyceride levels in shrimp plasma. Levels of triglyceride (triacylglycerol [TAG]) were measured in shrimp plasma during WSSV infection. As Fig. 8 shows, from 12 hpi to the end of the experiment the WSSV group had significantly lower TAG levels than the PBS controls.

Changes in virus copy number reach a peak between 12 and 24 hpi. After WSSV challenge, the number of copies of WSSV viral DNA in the pleopods of the challenged shrimp was quantitated using a TaqMan PCR. As Fig. 9A shows, the amount of accumulated WSSV DNA was initially very low, but after 12 hpi, virus quantity increased by ~ 350 times, and it continued to increase from ~ 75 WSSV genome copies per 10^4 host genome copies to $\sim 11,800$ WSSV genome copies per 10^4 host genome

FIG. 6. WSSV infection alters the levels of glucose and lactate in shrimp plasma. Glucose (A) and lactate (B) concentrations in plasma were measured at the indicated time points in WSSV-challenged shrimp versus PBS-injected controls. Statistical significance is indicated by single ($P < 0.05$) or double ($P < 0.005$) asterisks.

copies (~ 157 times) between 24 and 36 hpi. Figure 9B presents the results for SYBR green qPCR and shows that a similar pattern was found for the number of mRNA copies of the WSSV structural protein VP28. We note that the fold increase in WSSV copy number in Fig. 9A reached a peak at between 12 and 24 hpi, suggesting that 12 to 24 hpi was the interval during which viral replication occurred. Conversely, the subsequent reduction in fold increase between 24 and 36 hpi suggested that viral genome replication had ended. It is possible that by 36 hpi, a secondary infection cycle might have begun; we therefore took 24 hpi as the time point when the late stage of WSSV infection began. Taking these two time points, i.e., 12 and 24 hpi, to represent the beginning of the genome replication and late stages, respectively, is also consistent with previous reports that WSSV genome replication takes approximately 22 to 24 h (3, 35).

DISCUSSION

The data presented here suggest that WSSV relies on certain VDAC-related events to facilitate its infection process. Specifically, we propose that by ~ 12 h postinfection, i.e., during the viral genome replication stage of infection, metabolic changes

FIG. 7. WSSV infection increases glucose-6-phosphate dehydrogenase (G6PDH) activity in shrimp hemocytes at 12 hpi. G6PDH activity was measured at the indicated time points in WSSV-challenged shrimp versus PBS-injected controls. Statistical significance is indicated by single ($P < 0.05$) or double ($P < 0.005$) asterisks.

resembling the Warburg effect are induced in infected cells. The defining characteristic of the Warburg effect is that cells ferment glucose into lactate even under aerobic conditions (40). The first step of the glycolysis pathway is the conversion of glucose to glucose-6-phosphate (G6P) by hexokinase (HK). When hexokinase docks to the VDAC on mitochondria, it directly utilizes the energy generated from the mitochondria to facilitate this glycolysis; the aerobic glycolysis pathway in turn produces energy and leads to the accumulation of metabolite precursors that are essential for cellular metabolism and bioenergetics (19). The VDAC-bound HK also prevents the elevated levels of G6P from causing feedback inhibition and thus allows the aerobic glycolysis to continue even in the presence of sufficient energy and oxygen (19). Moreover, the interaction of HK and the VDAC prevents both the loss of mitochondrial membrane potential and mitochondrion-mediated cell death (14, 26). We found that the Warburg effect did not continue to the late stage of WSSV infection. During this stage, i.e., at ~24 hpi, we propose that increased expression of the VDAC caused a loss of mitochondrial membrane potential, which in turn led to MMP and cell death. Figure 9 shows evidence in support of a progression from the Warburg-like effect in the infected cells to subsequent cell death: the rate of increase in viral copy number is greatest between 12 and 24 hpi (Fig. 9A), i.e., during the viral genome replication stage, while a similar pattern is observed for the changes in copy number of the vp28 structural late gene (Fig. 9B). The timing of these events is also consistent with previous studies that have shown the WSSV replication cycle *in vivo* to be approximately 22 to 24 hpi, with the first expression of immediate-early genes at ~2 hpi, that of early genes from 6 to 18 hpi, and that of late (mostly structural) genes from 12 to 24 hpi (3, 35). Our models for these two phases are presented schematically in Fig. 10, and we discuss the evidence that supports our proposals in more detail below.

The VDAC is involved in the regulation of global mitochondrial function in response to cellular energy demand and stresses (13, 14). The VDAC is also instrumental in mitochondrial membrane permeabilization (MMP), which is a critical step in several cell death pathways (11). Some pathogens in-

FIG. 8. WSSV infection leads to a decrease in triglyceride levels in shrimp plasma. Plasma triglyceride concentrations were measured at the indicated time points in WSSV-challenged shrimp versus PBS-injected controls. Statistical significance is indicated by single ($P < 0.05$) or double ($P < 0.005$) asterisks.

teract with a variety of mitochondrial components to modulate MMP for their own benefit (1). For example, the influenza virus protein PB1-F2 and the hepatitis B virus X protein both induce MMP during the infection process (28, 41), whereas the vaccinia virus protein F1L inhibits both MMP and the release of cytochrome *c* (9). Our present data suggest that WSSV infection also interferes with mitochondrial activity: Fig. 4 shows that at 24 and 48 hpi, WSSV infection causes an increase in the number of cells with reduced mitochondrial membrane potential.

One of the most critical mitochondrial functions is energy metabolism, and this is exploited by viruses to benefit their replication process. For instance, in HeLa cells infected with vaccinia virus, ATP generation was increased by the virus-induced upregulation of two mitochondrial genes associated with energy production (2). When the upregulation of ATP was inhibited, fewer viral proteins and virus copies were produced. In the present study, within the first 12 h of infection there was no significant difference in ADP/ATP ratio between the PBS-treated group and the WSSV-injected group (Fig. 5). We hypothesize that in the WSSV group, even though more ATP energy is being produced by the aerobic glycolysis pathway (presumably via the threshold mechanism described by Vazquez et al. [33]), no net increase in energy was observed because the energy produced was simultaneously being utilized. Glycolysis, the citric acid cycle, and oxidative phosphorylation are tightly linked (7, 8), and a switch away from oxidative phosphorylation in the mitochondria is another characteristic of the Warburg effect (32, 40). Twelve hours later, at 24 hpi, enhanced aerobic glycolysis was no longer evident and there was a relative decline in ATP. In terms of the WSSV replication cycle in shrimp, which is about 22 to 24 hpi (3, 35), these results mean that the ADP/ATP ratio is low for the viral genome replication stage, while both a high ADP/ATP ratio and the loss of mitochondrial membrane potential (Fig. 4) are seen in the late stage (24 hpi). This progression appears to favor the virus, which would need cellular energy during the earlier phases of the replication cycle, after which it

FIG. 9. WSSV replication in shrimp. Histogram data represent the number of copies of WSSV genomic DNA (A) and WSSV VP28 mRNA (B) detected at the indicated time point, while the relative change from one time point (t_n) to the next (t_{n+1}) is defined as $(t_{n+1})/(t_n)$ and is shown by the dots.

would then benefit from MMP-triggered cell death because this would allow the virion particles to be released.

The Warburg effect is also associated with an increase in the flux of the pentose phosphate pathway (PPP) (10). This increased activity is seen in viruses as diverse as influenza virus, cucumber mosaic virus, and hepatitis C virus (in the early stage of infection) (6, 22, 29), and it helps to ensure that there is sufficient ribose-5-phosphate (R5P) for the synthesis of the nucleotides and nucleic acids necessary for viral genome replication. Glucose-6-phosphate dehydrogenase (G6PDH) is a key enzyme in the PPP, and the increase in G6PDH activity at 12 hpi (Fig. 7) suggests that a similar metabolic rerouting into the PPP also occurs during WSSV infection. Subsequently, at the end of the first replication cycle (24 hpi), there was no significant difference in G6PDH activity between the WSSV-

FIG. 10. Schematic representation of the *in vivo* metabolic changes in shrimp hemocytes at the viral genome replication stage (12 h) (A) and the late stage (24 h) (B) of the first WSSV replication cycle. Measured concentrations or activities are shown in rounded boxes: red indicates an increase and green indicates a decrease relative to the respective PBS control. Yellow boxes showed no change. VDAC expression level data are from Wang et al., (36), Wang et al., (37), and our unpublished data. Hexokinase mRNA expression levels at 24 hpi are from unpublished microarray data. No HK data are presently available for 12 hpi. Increases in pathway use are indicated by the thicker solid arrows. ψ_m represents membrane potential (no data are available for ψ_m at 12 hpi). The shaded box at the top of the 12-h schematic encompasses concentration changes that are consistent with the Warburg effect. The shaded box at the bottom left of the 24-h schematic shows changes associated with MMP.

injected group and the PBS-injected group (Fig. 7). We also observed another peak in G6PDH activity at 36 hpi, which would be in the middle of a putative secondary WSSV replication cycle (24 hpi to 48 hpi). We conclude that these fluctuations in G6PDH activity are most probably caused by the temporal demands of the WSSV replication cycle.

Another aspect of the Warburg effect is that it counteracts oxidative stress in the host cell and thus prevents cell damage and/or death (23). Oxidative stress is caused by an accumulation of reactive oxygen species (ROS), and it is seen in the pathogenesis of viruses such as hepatitis C virus (HCV), influenza virus, and human immunodeficiency virus (17, 25). Here we found that the concentration of hydrogen peroxide (H_2O_2) was significantly lower in the WSSV-infected shrimp at 12 hpi and significantly higher at 24 hpi (Fig. 5B). In cells exhibiting the Warburg effect, a reduction in H_2O_2 concentration results from changes in the activity of G6PDH and the pentose phosphate pathway, because during the generation of R5P, the PPP also generates a substantial amount of cytoplasmic NADPH. NADPH functions as a reducing agent in various cellular pathways, and it is also important for protection against oxidative stress (12, 34). The observed increase in G6PDH activity at 12 hpi (Fig. 7) might therefore have contributed to the observed decrease in H_2O_2 at this time (Fig. 5B). Conversely, when G6PDH activity returned to basal levels at 24 hpi, the H_2O_2 concentration became significantly higher than that in the PBS controls (Fig. 5B). We also note that the main source of ROS production is the mitochondria, and mitochondrial dysfunction often accompanies and increases oxidative stress (43). As discussed above, the unchanged ADP/ATP ratio at 12 hpi (Fig. 5A) does not imply that the mitochondria are still producing energy at normal levels; however, it seems likely that the mitochondria are still functional to the extent that they are able to convert at least some ADP to ATP at this time. Subsequently, the increased ADP/ATP ratio at 24 hpi (Fig. 5A) together with the JC-1 data in Fig. 4 suggest that mitochondrial function has become seriously disrupted by 24 hpi. This disruption would be expected to increase the levels of ROS production, and this is consistent with the low level of H_2O_2 that was observed at 12 hpi versus the higher level at 24 hpi.

Lastly, we note that, unlike for the other metabolites studied here, there was a significant and sustained decrease in plasma triglyceride levels from 12 hpi through to the end of the experiment (Fig. 8). During virus infection, triglycerides are used not only for energy production but also for the synthesis of macromolecules that are used in virion assembly (6, 16). However, presently it remains unknown which of these two accounts for the decrease in TAG after WSSV infection. More work will need to be done to determine whether the triglycerides are involved predominantly in energy production or lipogenesis after WSSV infection.

In conclusion, the evidence presented here suggests that a Warburg-like effect is induced in the target cells of WSSV-infected *L. vannamei* at ~12 hpi. This would favor the virus by helping to meet its high demand for cellular energy and basic building blocks during viral genome replication. The Warburg effect is associated with increased levels of the VDAC, and as expected, increases in the levels of this key factor (and several other critical metabolites) were seen here. The docking of hexokinase to the VDAC is also a critical element of the

Warburg effect, although unfortunately, so far we have not been able to confirm this experimentally in WSSV-infected cells. Subsequently, at 24 hpi, the metabolic pathways seem to shift from biosynthesis toward cell death. At the late stage of WSSV infection, there was a reduction in glucose consumption (Fig. 6A). This suggests that glycolysis was inhibited, which in turn would have had a limiting effect on downstream metabolic pathways. The consequences of high glucose levels in the plasma include mitochondrial dysfunction, the generation of ROS, loss of mitochondrial membrane potential, an increased ADP/ATP ratio, and finally the induction of cell death (24, 42). (The significantly reduced levels of glucose and lactate at 36 hpi and later were presumably the result of WSSV infection causing severe damage to the shrimp physiology.)

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