1 **Title page**

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3 **White spot syndrome virus infection induces Caspase 1-mediated antiviral cell**

4 **death in crustacean**

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24    **Abstract**

25    In vertebrates, pyroptosis is an intensely inflammatory form of programmed cell death which

26    is dependent on Caspase 1 activation and release of cytoplasmic cytokines including IL-1β. This

27    death pathway is critical for controlling pathogenic infection by mobilizing immune cells and

28    stimulating the development of adaptive immune response. In invertebrates, however, due to the

29    lack of adaptive immune response, it is still elusive whether Caspase 1-dependent cell death

30    pathway exists. In this study, our data showed that Caspase 1-mediated cell death was activated

31    by white spot syndrome virus (WSSV) infection to control the virus in shrimp. Caspase 1 had a

32    higher expression level in hemocytes and lymphoid-like organ in shrimp and WSSV infection

33    was significantly promoted upon the inhibition of Caspase 1 enzymatic activity. IL-1β-like

34    protein was identified as the substrate of Caspase 1 and its interaction with Caspase 1 was

35    validated ectopically and endogenously. Moreover, IL-1β like protein was released into

36    extracellular contents under WSSV infection and Prophenoloxidase system was activated,

37    resulting in the reduction of WSSV copies *in vivo*. Our data unraveled a previously unidentified

38    mechanism through which Caspase 1-dependent cell death controlled virus infection in shrimp.

39    Therefore, our study opened the possibility that an invertebrate cytokine network might be

40    operative and regulate host defenses against virus infection as in vertebrates.

41    Key words: shrimp; virus; pyroptosis; Caspase 1; IL-1β-like protein

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47 **Author Summary**

48    The elimination of infected cells by programmed cell death is considered as one of the most

49    important anti-pathogen responses. Pyroptosis is one form of programmed cell death, which is

50    mediated by Caspase 1 activation. Activated Caspase 1 can process the inactive precursors of

51    interleukin 1β (IL-1β) into mature inflammatory cytokines to induce potent immune responses.

52    Meanwhile, pyroptosis also induces morphologic changes, DNA fragmentation and chromatin

53    condensation to destroy infected cells. However, due to the lack of efficient adaptive immune

54    responses, it is still unclear whether Caspase 1-mediated cell death also exits and plays such an

55    important role in defending hosts from pathogen invasion in invertebrates. In our data, Caspase 1

56    gene was characterized and Caspase 1-mediated cell death pathway was validated in shrimp. The

57    Caspase 1-mediated cell death pathway was enhanced by white spot syndrome virus (WSSV)

58    infection to control the virus in shrimp. IL-1β-like protein was cleaved by Caspase 1 and released

59    into extracellular contents under WSSV infection to activate Prophenoloxidase system, leading

60    to the reduction of WSSV copies in shrimp in vivo. On the other hand, silencing of *Caspase 1* or

61    *IL-1β-like* gene facilitated virus infection in shrimp. Our study indicated that Casapase-1-

62    mediated cell death played an important antiviral role in shrimp against WSSV infection and

63    opened the possibility that an invertebrate cytokine network might be operative.

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70 **Introduction**

71    One of the most important anti-pathogen responses consists of the elimination of the infected

72 cells by programmed cell death, a response found in all metazoans (1, 2). Apoptosis is perhaps

73 the most widely recognized programmed cell death, and is defined by the requirement for

74 particular cysteine-dependent aspartate-specific proteases, commonly Caspases 3/7, which

75 produce an orchestrated disassembly of the cell resulting in mounting more severe immune

76 responses (3, 4). It is found that apoptosis plays a very important role in determining the outcome

77 of host-pathogen interactions (5, 6). Other than Caspase 3/7-mediated apoptosis, pyroptosis is a

78 more recently identified pathway of host cell death that is stimulated by a range of pathogen

79 infections and non-infectious stimuli (7, 8).

80    Unlike apoptosis, pyroptosis occurs after Caspase 1 activation. Caspase 1 is usually activated

81 by protein complexes termed inflammasomes in mammals (9). Two types of inflammasomes

82 formed by Nod-like receptors (NLRs) have been identified. One type is NLRC4 and murine

83 NLRP1b and the other type is NLRP3 (10). NLRC4 and murine NLRP1b contain CARD

84 domains that directly interact with the Caspase 1 CARD and then activate Caspase 1 proteolytic

85 activity. In contrast, NLRP3 contains a Pyrin signaling domain instead of a CARD domain (11).

86 The Pyrin domain binds the Pyrin domain of the adaptor protein ASC which is composed of only

87 a Pyrin and a CARD domain. ASC then recruits Caspase 1 via CARD-CARD interactions and

88 finally activate Caspase 1 proteolytic activity (12). Activated Caspase 1 can recognize and

89 process the inactive precursors of interleukin 1β (IL-1β) into mature inflammatory cytokines,

90 which is then secreted into intracellular contents (12). IL-1β is an important mediator of the

91 inflammatory response of the host against pathogen infection (13). Meanwhile, pyroptosis also

92    induces morphologic changes, DNA fragmentation and chromatin condensation to destroy

93    infected cells (7, 9).

94    During pathogen infection, the host benefits from pyroptosis. The compromised cells are

95    eliminated and more severe immune responses are launched, effectively destroying the protective

96    environment where infectious agents can thrive (14). In Caspase-1-deficient macrophages where

97    pyroptosis is compromised, higher intracellular bacterial loads are detected during *L.*

98    *pneumophila* infection (14). Pyroptosis also promotes pathogen clearance by acting as an alarm

99    signal that recruits immune cells to the site of infection (15). The secretion of IL-1β and IL-18

100   promotes leukocyte activation and immuno-stimulatory factors are released from lysed cells into

101   the extracellular milieu (15). A bunch of released cytosolic products are potent damage-

102   associated molecular patterns (DAMPs), such as high-mobility group box 1 (HMGB1), heat-

103   shock proteins and DNA-chromatin complexes. By the activation of pattern-recognition

104   receptors, these DAMPs accelerate proinflammatory cytokine production (16). On the other hand,

105   pathogens have evolved effector proteins capable of directly inhibiting Caspase 1 and

106   inflammasome activation, preventing cells from pyroptosis (17, 18). Virulent *Pseudomonas*

107   strains express the effector protein ExoU, which blocks Caspase 1 activation and *Y.*

108   *pseudotuberculosis* T3SS Rho-GTPase activating protein YopE inhibits inflammasome

109   activation (17, 19). The host-pathogen interaction mechanisms suggest that the activity of

110   Caspase 1 leading to pyroptosis may represent an important antiviral pathway that prevents

111   replication and spread of viruses to neighboring cells.

112   Pyroptosis is such a potent mechanism to clear intracellular pathogens. However, current

113   studies on the role of pyroptosis in host-virus interactions has not been extensively explored and

114   it is still unclear whether pyroptosis also exits and plays such an important role in defending

115   hosts from pathogen invasion in invertebrates. In this study, we aim to find out whether

116   pyroptosis is also one of the pathways that contributes to eliminating virus in invertebrate. The

117   results revealed that the activity of Caspase 1 in shrimp was enhanced by white spot syndrome

118   virus (WSSV) infection. The silencing of *Caspase 1* or *IL-1β-like* gene facilitated virus infection

119   in shrimp. Our study indicated that Casapase-1-mediated cell death played an important antiviral

120   role in invertebrate.

121   **Results**

122   **Involvement of Caspase 1 in the virus infection to shrimp**

123   In order to investigate whether Caspase 1-mediated cell death plays a role in the immune

124   response of invertebrates to virus infection, the profiles of *Caspase 1* gene was characterized in

125   the course of shrimp immunity against WSSV infection. The sequence analysis revealed that the

126   shrimp Caspase 1 contained caspase activity domains (Fig 1A). The expression of the gene in

127   different organs was then examined by real-time PCR. The result showed that Caspase 1

128   expression was most significant in hemocytes followed by lymphoid-like-organ, however, we

129   can barely detect the mRNA of Caspase 1 in muscle and heart (Figure 1B). The expression

130   pattern of the gene was further confirmed by detection of protein levels as shown in Figure 1C.

131   These results indicated the shrimp Caspase 1 might function in immunity system--hemocytes and

132   lymphoid-like organ in shrimp, where fighting off invading pathogens usually took place, so the

133   result indicated that Caspase 1 might play a role in the antiviral cellular pathway..

134   To explore the role of Caspase 1 in virus infection to shrimp, the expression of Caspase 1

135   was detected at different time points after shrimp were infected with WSSV. The results revealed

136   that the transcriptional level of Caspase 1 was significantly upregulated with the prolonged

137   infection time (Fig 1D). Meanwhile, the protein level of the gene also showed similar expression

138    patterns during WSSV infection (Figure 1E).Taken together, these results indicate that Caspase-

139    1-mediated cell death is involved in virus infection of shrimp.

140    **Requirement of enzymatic activity of Caspase 1 in virus infection**

141    To determine whether the enzymatic activity of Caspase 1 was associated with virus

142    infection, shrimp were challenged with increasing concentrations of WSSV and hemocytes were

143    collected for the detection of Caspase 1 activity by incubating with the substrate Ac-YVAD-pNA.

144    The results showed that Caspase 1 activity was greatly enhanced while more viruses were used

145    for infection (Fig 2A). Moreover, Caspase 1 activity increased dramatically along with the

146    prolonged infection time (Fig 2B). These results indicated that the enzymatic activity of Caspase

147    1 was actually indispensible in the pathway of virus infection.

148    To explore whether the Caspase 1 activity facilitated or inhibited virus infection, Caspase 1

149    inhibitor Belnacasan was used in combination with WSSV injection or not, and WSSV copy

150    number was then measured by quantitative real-time PCR. The data showed that the WSSV copy

151    number increased significantly upon the inhibition of  Caspase 1 enzymatic activity, suggesting

152    that Caspase 1 played an antiviral role possibly conducted by its enzymatic activity.

153    **Interaction between Caspase 1 and IL-1β like protein**

154    In order to reveal the cellular pathway in which Caspase 1 participated to play an antiviral

155    role, we performed Coimmunoprecipitation (Co-IP) to identify the substrate of Caspase 1. The

156    Co-IP data showed that one specific band was discovered after Caspase 1- specific antibody but

157    not IgG was used for CoIP, which was then identified as IL-1β like protein by mass spectrometry

158    (Fig 3A). To further demonstrated the interaction between Caspase 1 and IL-1β like protein,

159    Flag-Caspase 1 and His-IL-1β like protein was respectively cloned into PIZ/V5-His plasmid and

160    co-transfected into insect High Five cells. Anti-Flag IP was performed and then proteins were

161    analyzed by Western blotting. The result indicated that His-IL like protein was actually detected

162    (Fig 3B), showing the interaction between Caspase 1 and IL-1β like protein. Meanwhile, anti-His

163    IP was also performed and Flag-Caspase 1 could also be found by Western blotting (Fig 3C).

164    These results revealed that the two proteins could interact with each other by their expressions in

165    insect cells.

166        To further confirm the interaction Caspase 1 and IL-1β like proteins *in vivo*, endogenous IP

167    was performed using Caspase 1 or IL-1β like protein antibody. The results showed that IL-1β

168    like protein was detected by Caspase 1 endogenous IP and vice versa (Fig 3D).

169        Taken together, these findings identify IL-1β like protein is the substrate of Caspase 1 and

170    the two proteins could interact with each other.

171    **Redistribution of IL-1β like protein induced by WSSV infection to activate phenoloxidase**

172    **system**

173        To investigate the functions of Caspase 1 and IL-1β like protein interaction *in vivo*, siRNA

174    respectively targeting *Caspase 1* gene and *IL-1β like protein* gene was designed. The

175    effectiveness of siRNAs was examined by quantitative real-time PCR and Western blotting.

176    From the detected mRNA and protein level, it could be concluded that the siRNAs efficiently

177    knocked down the expressions of *Caspase 1* and *IL-1 like protein* genes (Fig 4A).

178        As reported in mammary cells, IL-1β is firstly interacted with and then cleaved by Caspase 1

179    before it is released into extracellular contents (20), however, in this study, it is still elusive how

180    IL-1β like protein reacts upon virus infection. Therefore, the level of IL-1β like protein in

181    hemocytes and the corresponding plasma before and after WSSV infection was examined.

182    Hemolymph was collected and centrifuged to separate the hymocytes and plasma for the

183    detection of IL-Iβ like protein. Western blotting analysis revealed that the expression level of IL-

184  1β like protein was increased in the plasma and decreased in hemoctes when shrimp were

185  challenged with WSSV, indicating that IL-1β like protein was released into extracellular contents

186  under virus infection (Fig 4B). In order to determine whether Caspase 1 mediated the release of

187  IL-1 like protein, the expression of Casapse 1 was firstly knocked down by siRNA and then the

188  expression pattern of IL-1β like protein was examined. The results showed that the level of IL-1β

189  like protein in the plasma decreased while the level in the hemocytes increased upon infection

190  with WSSV (Fig 4C). These data showed that the release of IL-1β like protein into plasma was

191  controlled by Caspase 1 in shrimp.

192  To further determine whether IL-1β like protein was required to be cleaved by Caspase 1

193  before releasing into extracellular contents, the enzymatic activity of Caspase 1 was inhibited by

194  Belnacasan and then the level of IL-1β like protein was examined. The result showed that the

195  level of IL-1β like protein didn't increase in the plasma, suggesting that the enzymatic activity of

196  Caspase 1 was required for the release of IL-1β like protein and that IL-1β like protein needed

197  cleavage by Caspase 1 before releasing into extracellular contents (Fig 4C).

198  Prophenoloxidase (PO) activating system is an important identification and defense system

199  for eliminating viruses in crustaceans (21). To find out whether Caspase 1 mediated cell death

200  could influence PO system, Caspase 1 and IL-1β like protein were respectively knocked down

201  and then the PO activity was measured with or without WSSV challenge. As expected, WSSV

202  challenge greatly induced the PO activity (Fig 4D). However, either Caspase 1 knockdown or IL-

203  1β like protein knockdown partially inhibited the PO activation (Fig 4D), suggesting that

204  Caspase 1 mediated cell death could enhance PO activity upon WSSV infection.

205    Taken the above data together, these findings demonstrate that the releasing of IL-1β like

206    protein is dependent on the enzymatic activity of Caspase 1, resulting in the activation of PO

207    system and cell damage which prevent shrimp from virus infection.

208    **Negative correlation of Caspase 1 and IL-1β like protein expressions with virus infection**

209    To investigate the influence of Caspase 1-mediated cell death on virus infection, WSSV copy

210    number was detected upon knockdown of either *Caspase 1* or *IL-1β like protein* gene in shrimp.

211    The results showed that the silencing of Caspase 1 or IL-1β like protein significantly increased

212    the copy number of WSSV compared to the control, indicating that Caspase 1-mediated cell

213    death played an important antiviral role in shrimp immunity (Fig 5A), which was further

214    demonstrated by the shrimp cumulative mortality analysis (Fig 5B).

215     Collectively, these findings reveal a novel pathway in which Caspase 1-mediated cell death

216    induces PO activation and plays a critical antiviral role in shrimp immunity (Fig 5C).

217    **Discussion**

218    The outbreak of viral diseases is a major concern preventing the development of shrimp

219    aquaculture industry. WSSV is one of the most virulent shrimp viruses and the processes of

220    pathogenesis are extremely complex. Due to the lack of effective therapeutics to control virus, it

221    is of great importance to understand viral pathogenesis and host responses at the molecular level

222    in order to prevent virus invasion. So far, some studies have demonstrated that many genes and

223    pathways are involved in host defense against WSSV, including Ran protein, the Ras-activated

224    endocytosis process, the RNA interference pathway and apoptosis (22-25). Apoptosis is maybe

225    the best described form of cell death that is mediated by the activation of the apoptotic caspase

226    enzymes Caspase 3/7 and the antiviral role of apoptosis has been discovered both in invertebrates

227    and vertebrates. Currently, pyroptosis, which is mediated by Caspase 1 activation, is thought to

228     have the capacity of eliminating viruses only in vertebrates (26). In vertebrates, pyroptosis

229     activation can result in not only the production of activated inflammatory cytokines, but also

230     rapid cell death characterized by plasma-membrane rupture and release of proinflammatory

231     intracellular contents(26). On the other hand, viruses including HIV have evolved mechanisms to

232     inhibit pyroptosis, enhancing their ability to persist and cause disease (27). Ultimately, it is the

233     competition between host and pathogen to regulate pyroptosis, and the outcome dictates life or

234     death to the host or to viruses. Based on the critical role of Caspase 1 in the defense of a wide

235     range of virus infections in vertebrates, in this study, Caspase 1 gene was first cloned from

236     shrimp and the activity of Caspase 1 was then characterized. Our data showed that Caspase 1

237     activity was upregulated upon WSSV infection and the knockdown of Caspase 1 facilitated virus

238     infection. Compared with the function of pyroptosis in vertebrates, our study revealed that

239     Caspase 1-mediated cell death also contributed to eliminating virus in crustacean. Our findings

240     indicated that the antiviral function of Caspase 1 was conservative although the sophisticated

241     immune responses of shrimp have not been developed yet.

242         In vertebrates, the activated Caspase 1 recognizes and cleaves IL-1β into the mature form.

243     The release of mature IL-1β further enhances the inflammatory response by stimulating immune

244     cell activation and more cytokine secretion (28), leading to critical physiological consequences

245     such as fever and hypotension. However, the cleavage and release of IL-1β is not indispensible

246     for caspase 1-mediated cell death as demonstrated by IL-1β-/- knockout animals (29). In our

247     study, the substrate of Caspase 1, which was named IL-1β like protein, was firstly identified. The

248     results showed that WSSV infection induced the redistribution of IL-1β like protein into plasma

249     of shrimp, indicating that IL-1β like protein was released into extracellular contents upon virus

250     infection. The released IL-1β like protein, instead of triggering inflammation responses, activated

251 phenoloxidase system of shrimp, an important invertebrate defense mechanism against virus

252 infection. These data demonstrated that the function IL-1β like protein participating in Caspase

253 1- mediated cell death in shrimp was similar with that in vertebrates, and that IL-1β like protein

254 functioned as cytokine-like molecules in invertebrates. Although invertebrates including shrimp

255 lack adaptive immune system, several putative functional analogues of inflammatory cytokines

256 have been identified so far. For example, tumor necrosis factor (TNF)-like molecules and

257 interleukin-1 (IL-1)-like molecules are detected in a variety of invertebrates, such as insects,

258 echinoderms and protochordates (30, 31). Moreover, chemokine IL-8 and transforming growth

259 factor β1 are vertebrate cytokines involved in inflammatory processes and molecules cross-

260 reacting with the two proteins using antibodies have been identified in mollusks (32). In addition,

261 IL-2-like activity is detected in protochordates and echinoderms which have hematopoietic

262 organs and T-like cells (33). In this study, our data suggested that an invertebrate cytokine

263 network might be operative and regulate host defense mechanisms against virus infection as in

264 vertebrates.

265 **Material and methods**

266 **Shrimp culture and WSSV challenge**

267 *Marsupenaeus japonicas* shrimp was cultured in groups of 20 individuals in tanks containing

268 aerated seawater at room temperature. The individual shrimp was about 10g in weight and 10 to

269 12 cm in length. To ensure the absence of WSSV in shrimp prior to experimental infection, PCR

270 was performed using WSSV specific primers (5'-TATTGTCTCTCCTGACGTAC-3' and 5'-CA

271 CATTCTTCACGAGTCTAC-3'). Virus-free shrimp were infected with WSSV ($10^5$ copies/ml)

272 by injection into the lateral area of the fourth abdominal segment. Three shrimp were randomly

273   collected for each treatment. At different time post-infection, the shrimp hemolymph was

274   collected for later use.

**Rapid amplification of cDNA ends (RACEs)**

276   Total RNAs were extracted from shrimp hemocytes using mirVana miRNA™ Isolation Kit

277   (Ambion, USA). RACE experiments were conducted using 5'-3' RACE Kit (Roche, USA)

278   according to the manufacturer's protocol. Briefly, cDNA was synthesized and applied for PCR

279   and nest-PCR. PCR was performed using 3' RACE primer, 5' RACE primer or/and gene-

280   specific primers. The amplified DNAs were sequenced.

**Quantitative real-time PCR for detections of virus copies and gene expression**

282   Quantitative real-time PCR was performed to quantify WSSV copies and gene expression

283   level in shrimp. DNA or RNA was extracted from shrimp hemocytes using SQ tissue DNA kit

284   (Omega-BioTek, USA) or RNA Isolation kit (Ambion, USA). RNA was reversely transcribed

285   into cDNA by cDNA synthesis kit (Takara, Japan).

286   The WSSV copies were evaluated with WSSV-specific primers (5'-TTGGTTTCAG

287   CCCGAGATT-3' and 5'-CCTTGGTCAGCCCCTTGA-3'). The gene expression level was

288   determined using gene-specific primers (Caspase 1, 5'-TTTCAAGGCTGACCAGT GCCCTAC-

289   3' and 5'-ACCTGCGTCAAGACCCTCACCTC-3'; IL-1β, 5'- GCGTTCC

290   ACATTTCAGATACTCGG-3' and 5'-TGTAGACCATTCCAAAGGCTCCC-3'). The 25μL

291   PCR solution contained 12.5μL of 2×Premix Ex Taq (TaKaRa, Japan), 0.5μL of 10μM forward

292   primer, 0.5μL of 10μM reverse primer and 1μM DNA template. The stages of PCR program

293   were 95°C for 5 min, followed by the amplification stage consisting of 40 cycles of 95°C for 10 s

294   and 60°C for 30 s.

**Western blot analysis**

296     Proteins were separated by a 12% SDS-polyacrylamide gel, which were then transferred onto

297     a polyvinylidene difluoride (PVDF) membrane (Millipore, USA). The membrane was blocked

298     with 5% nonfat milk in phosphate buffered solution (PBS) for 2 h at room temperature. The blot

299     was incubated with a primary antibody at 4°C overnight. After washes with PBS, the blot was

300     incubated with the fluorescence-labeled anti-mouse IgG (Cell Signaling Technology, USA) for 2

301     h at 4°C. The blot was detected by Odyssey (Licor, USA) for signals.

**Caspase 1 activity detection**

303     Caspase 1 Activity Assay Kit was used to detect Caspase 1 activity according to the

304     manufacturer's instructions (Beyotime, China). Briefly, shrimp hemocytes were collected and

305     washed with PBS. Then, the hemocytes were lysed in 100μL ice-cold lysis buffer for 15 min and

306     centrifuged at 15,000×g (4°C) for 10 min. The supernatant was incubated with the substrate Ac-

307     YVAD-pNA (2mM) for 2 h at 37°C. The reaction was detected by a spectrophotometer at $OD_{405}$.

308     Caspase 1 inhibitor Belnacasan was used to inhibit Caspase 1 activity in shrimp. The inhibitor

309     was injected into shrimp at 100μg/g shrimp in combination with WSSV injection or not.

**Cell culture and plasmids transfection**

311     Insect High Five cells (Invitrogen, USA) were cultured and maintained at 27°C in Express

312     Five serum-free medium (Invitrogen) containing L-glutamine (Invitrogen). Cells were co-

313     transfected with plasmids expressing Flag-Caspase 1 and His-IL-1β like protein according to the

314     manufacture's instruction. Briefly, when the cells reached about 70% confluence in a 6-well

315     plate, 2μg for each indicated plasmid and 6μl Cellfectin Reagent were diluted in 100 μl Grace's

316     medium, respectively. The diluted plasmids and Cellfectin Reagent were then mixed gently and

317     incubated for 30 minutes at room temperature. The mixture was added to the cells and incubated

318    in a 27°C incubator for 5 h before the medium was replaced by fresh Express Five serum-free

319    medium. Cells were finally subjected to further research 48 h after transfection.

320    **Co-immunoprecipitation**

321    Cells were collected and lyzed in ice-cold lysis buffer (50mM Tris-HCl, 150mM NaCl, 0.2%

322    NP40, 1mM PMSF, 1×protease inhibitor, pH 8.0). The lysate was then centrifuged three times

323    for 5 min each to remove all the undissolved contents. The supernatant was incubated with

324    indicated antibody overnight at 4°C. Protein A+G agarose beads (Invitrogen, USA) were

325    incubated with the lysate for 2 h at 4°C. After washed three times with ice-cold wash buffer

326    (50mM Tris-HCl, 200mM NaCl, 0.1% NP40, 1mM PMSF, 1×protease inhibitor, pH 8.0), the

327    immuno-complexes were eluted by 100 mM glycine and subjected to Western blotting for the

328    detection of indicated proteins.

329    **Synthesis of siRNAs and RNAi assay**

330    Based on the sequences of *Caspase 1* and *IL-1β like protein* genes, siRNAs were designed to

331    specifically target the genes and then were synthesized in vitro using a commercial kit according

332    to the manufacturer's instructions (TaKaRa, Japan). The siRNAs used were Caspase 1-siRNA

333    (5'-AAACCACUCGGAAUGCGAUGCG-3')          and          IL-1β-like-protein-siRNA          (5'-

334    GGGAAGGCTTTGAGGTCCTTGT-3'). The scrambled siRNA not targeting any genes was

335    used as the control. The formation of double-stranded RNAs was monitored by determining the

336    size in agarose gel electrophoresis. The synthesized siRNAs were dissolved in siRNA buffer (50

337    mM Tris-HCl, 100 mM NaCl, pH 7.5) and quantified by Nanodrop 2000. The RNA interference

338    (RNAi) assay was conducted in shrimp by the injection of a siRNA into the lateral area of the

339    fourth abdominal segment at 30 μg/shrimp using a syringe with a 29-gauge needle. The siRNA

340    (15μg) and WSSV ($10^5$ copies/ml) were co-injected into virus-free shrimp at a volume of 100μL

341    per shrimp. At 12 h after the co-injection, the siRNA (15μg) (100μL /shrimp) was injected into

342    the same shrimp. For each treatment, 20 shrimp were used. At different times after the last

343    injection, the shrimp hemocytes were collected. Three shrimp specimens from each treatment,

344    randomly selected, were collected for analysis. The assays were biologically repeated for three

345    times.

**Phenoloxidase activity detection**

347    Hemolymph was centrifuged and the plasma was used for the detection of phenoloxidase

348    activity. The reaction buffer (0.1M K3PO4, 0.01M L-dopa, pH 6.0) and plasma was mixed and

349    then incubated at 28°C for 2 min. The activity of phenoloxidase was measured by a

350    spectrophotometer at OD492.

**Shrimp mortality analysis**

352    Shrimp with different treatments were cultured at 20 shrimp/treatment. Shrimp mortality was

353    examined every day. The experiment was biologically repeated three times.

**Statistical analysis**

355    The data from three independent experiments were analyzed by one-way analysis of variance

356    (ANOVA) to calculate the mean and standard deviation (SD) of the triplicate assays.

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## Figure legends

**Fig 1. Involvement of Caspase 1 in the virus infection to shrimp.** (A) Sequence analysis of shrimp Caspase 1. *Casapse 1* gene was cloned from shrimp hemocytes. Shrimp Caspase 1 contained three domains including active site, proteolytic cleavage site and dimer interface. (B) Detection of Caspase 1 mRNA in different tissues of shrimp. The Caspase 1 mRNA was examined by quantitative real-time PCR. Shrimp β-actin was used as an internal control. (C) Examination of Caspase 1 protein in different shrimp tissues. The Caspase 1 protein was detected by Western blot. Shrimp β-actin was used as an internal control. (D) Expression level of Caspase 1 mRNA in shrimp challenged with virus. Shrimp were challenged with WSSV. At different time post-infection, the Caspase 1 mRNA of shrimp hemocytes were analyzed by quantitative real-time PCR (*, $p<0.01$). (E) Determination of Caspase 1 protein level in WSSV-infected shrimp. Proteins were extracted from virus-infected shrimp hemocytes and analyzed by Western blot. The numbers indicated the time post-infection. Shrimp β-actin was used as an internal control.

**Fig 2. Requirement of enzymatic activity of Caspase 1 in virus infection.** (A) Influence of WSSV concentration on Caspase 1 activity. Caspase 1 activity was measured at $OD_{450}$ after shrimp were challenged with increasing WSSV concentration. Caspase 1 activity was detected at 48 h post-infection. (B) Effects of virus infection on Caspase 1 activity. Caspase 1 activity was measured at different time points after shrimp were challenged with WSSV. (C) Impact of inhibition of Caspase 1 activity on virus infection. WSSV copy number was measured by quantitative real-time PCR at 48 h after shrimp were treated with Caspase 1 inhibitor with or without WSSV infection.

474 **Fig 3. Interaction between Caspase 1 and IL-1β like protein.** (A) SDS-PAGE analysis of the

475 elute from Caspase 1 IP by silver staining. Caspase 1 IP was performed and the

476 immunoprecipitate was analyzed by SDS-PAGE by silver staining. The proteins were identified

477 by mass spectrometry. (B) Validation of Flag-Caspase 1 and His-IL-1β like protein by Anti-Flag

478 IP in insect High Five cells. The Flag-Caspase 1 and His-IL-1β like protein constructs were co-

479 transfected into insect cells and Anti-Flag IP was performed. The elute was analyzed by Western

480 blot. β-tubulin was used as an internal control. (C) Validation of Flag-Caspase 1 and His-IL-1β

481 like protein by Anti-His IP in High Five cells. Flag-Caspase 1 and His-IL-1β like protein were

482 cotransfected into High Five cells and Anti-His IP was performed and the elutes was analyzed by

483 western blot. β-tubulin was used as a control. (D) Endogenous Caspase 1 and His-IL-1β like

484 protein interaction by IP. Caspase 1 or IL-1β like protein IP was performed respectively and the

485 immunoprecipitates were analyzed by Western blot. β-tubulin was used as an internal control.

486 **Fig 4. Redistribution of IL-1β like protein induced by WSSV infection to activate**

487 **phenoloxidase system.** (A) Detection of Caspase 1 and IL-1β like protein knockdown efficiency.

488 The mRNA and protein levels of Caspase 1 and IL-1β like protein were respectively detected by

489 quantitative real-time PCR (left) and Western blot (right) at 48 h after shrimp were injected with

490 indicated siRNAs. (B) Detection of IL-1β like protein expression in hemocytes or plasma after

491 WSSV infection. The expression level of IL-1β like protein in hemocytes or plasma was

492 examined by Western blot with or without WSSV infection. Samples were collected at 48 h after

493 WSSV infection. (C) Evaluation of IL-1β like protein expression in hemocytes or plasma after

494 WSSV infection with or without Caspase 1 inhibition. The expression level of IL-1β like protein

495 in hemocytes or plasma was detected by Western blot after WSSV-infected shrimp were treated

496 with Caspase 1 siRNA or inhibitor. Caspase 1 inhibitor or siRNA was immediately injected into

497    shrimp after WSSV infection. Samples were collected 48 h after injection. (D) Examination of

498    phenoloxidase activity upon knockdown of Caspase 1 or IL-1β like protein in

499    shrimp. Phenoloxidase activity was measured at $OD_{492}$ after shrimp were treated with either

500    Caspase-1-siRNA or IL-1β-like-protein-siRNA at 48 h after WSSV injection.

501    **Fig 5. Negative correlation of Caspase 1 and IL-1β like protein expressions with virus**

502    **infection.** (A) Detection of WSSV copies after knockdown of Caspase 1 or IL-1β like protein

503    genes. WSSV copy number in Caspase-1-siRNA or IL-1β-like-protein-siRNA treated shrimp

504    was measured by quantitative real-time PCR. (B) Monitoring of shrimp cumulative mortality

505    upon knockdown of Caspase 1 or IL-1β like protein genes. (C)  A model for the Caspase 1-

506    mediated antiviral pathway in shrimp.

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520 **Fig 1**

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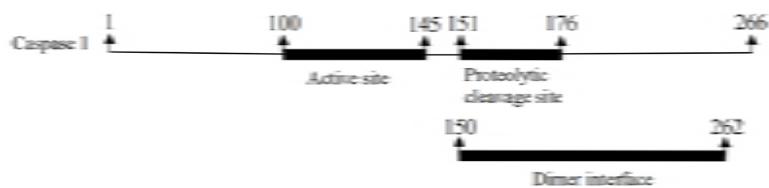


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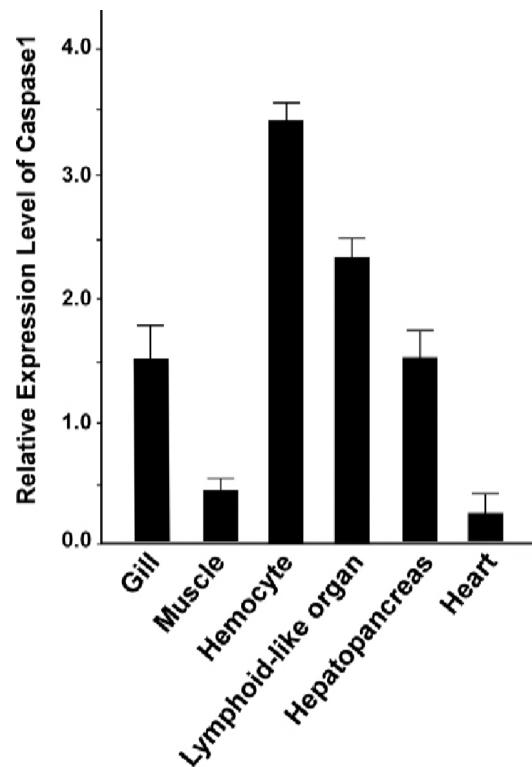


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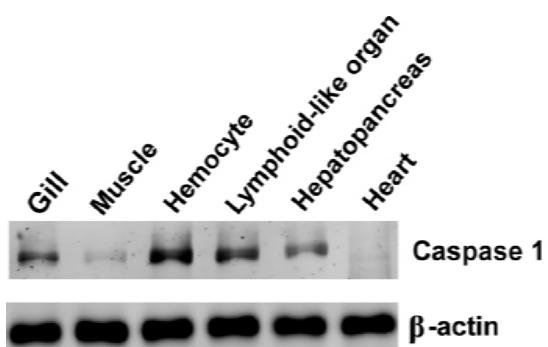


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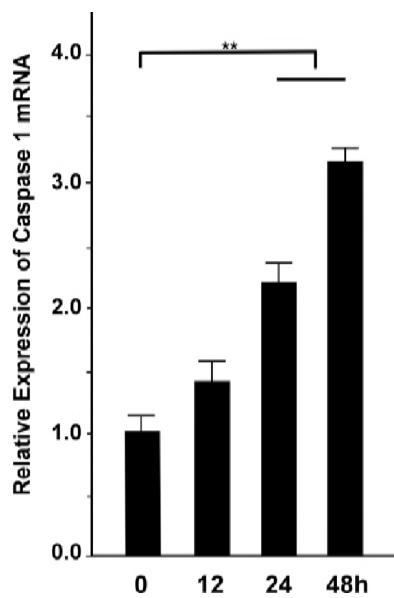


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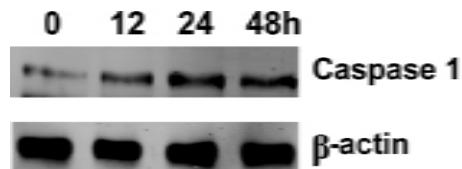


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541 **Fig 2**

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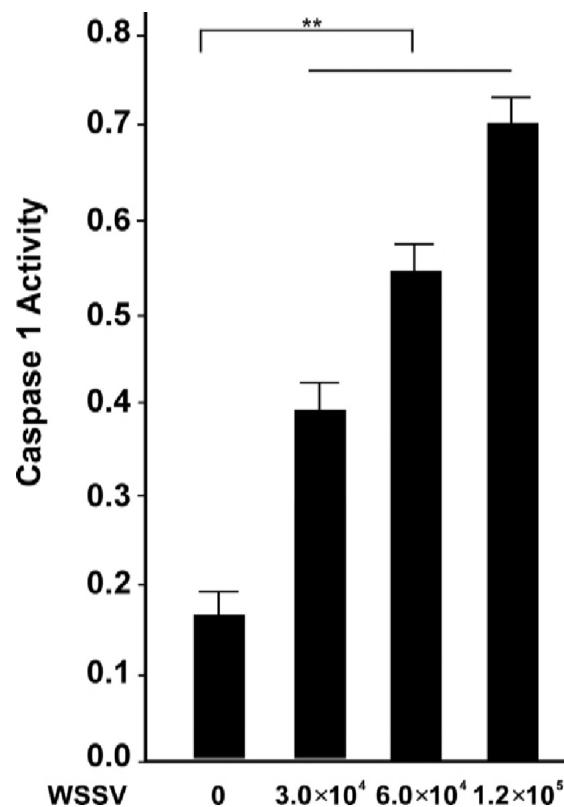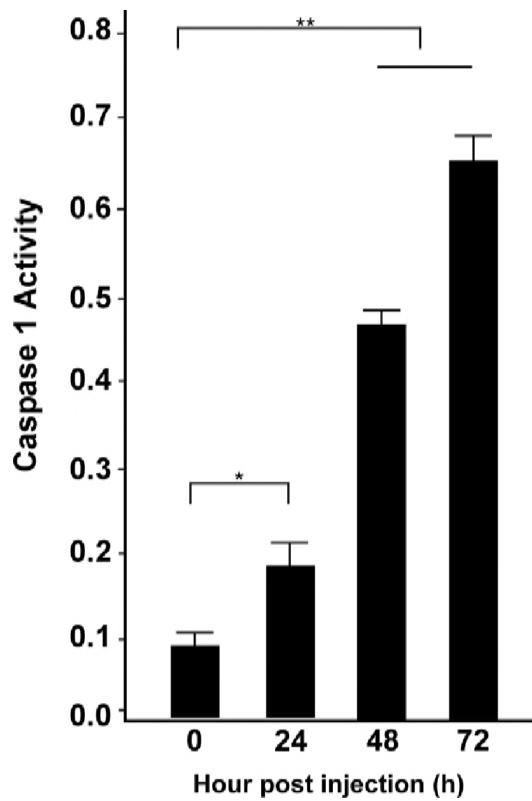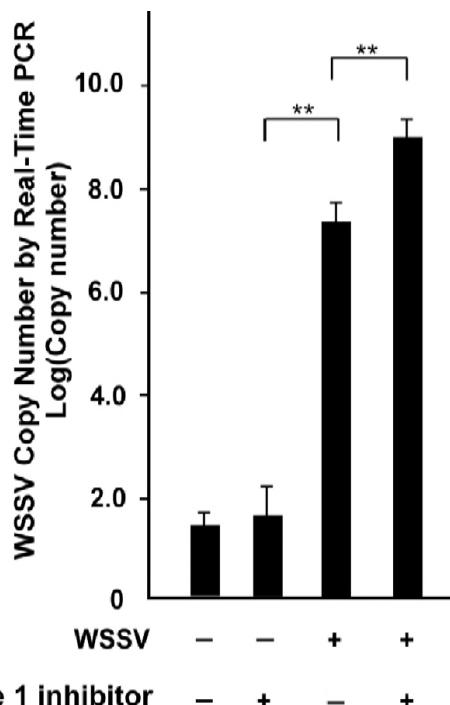
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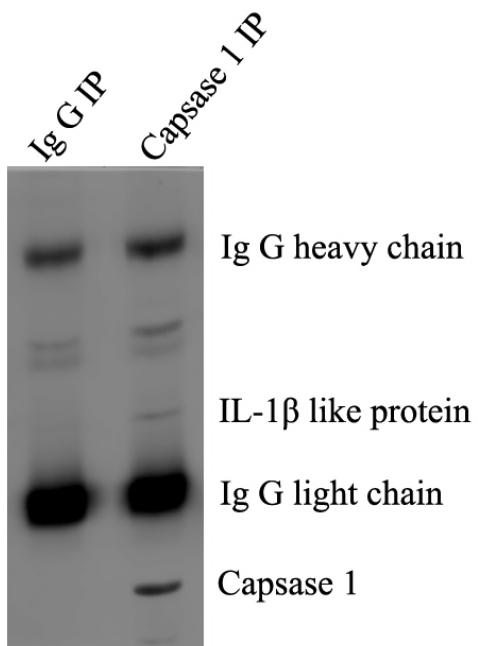


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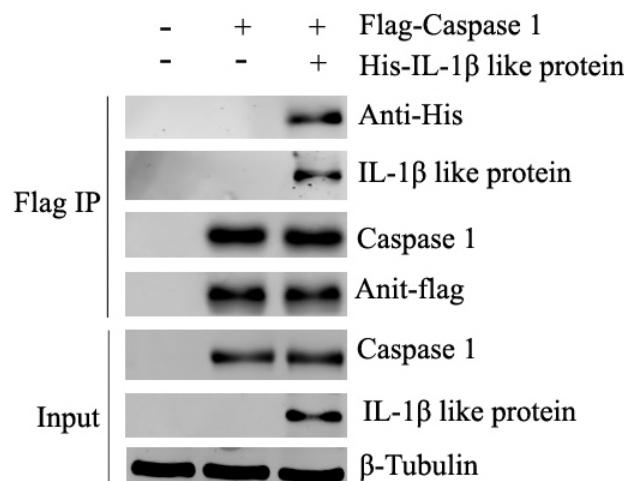


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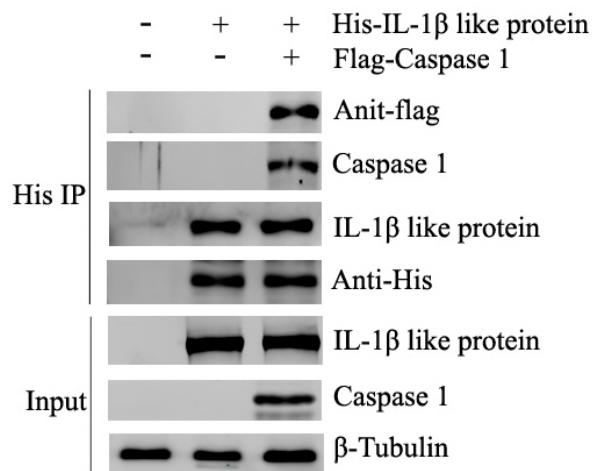


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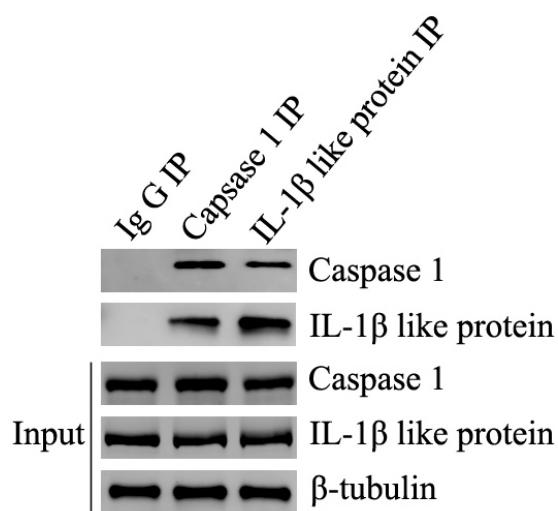


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578 **Fig 4**

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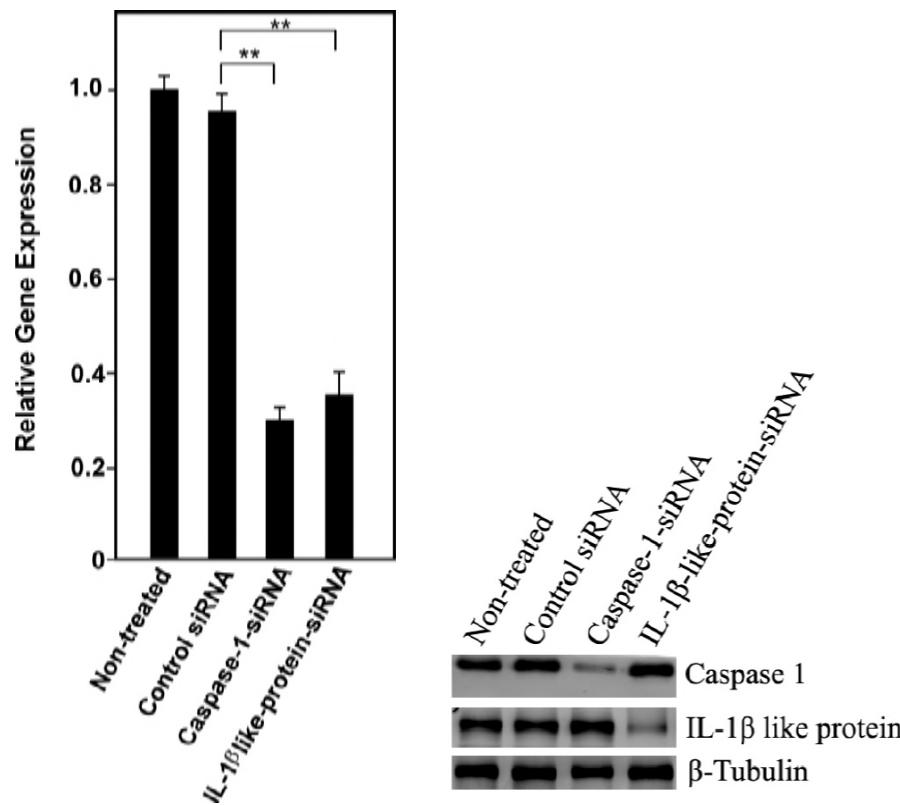


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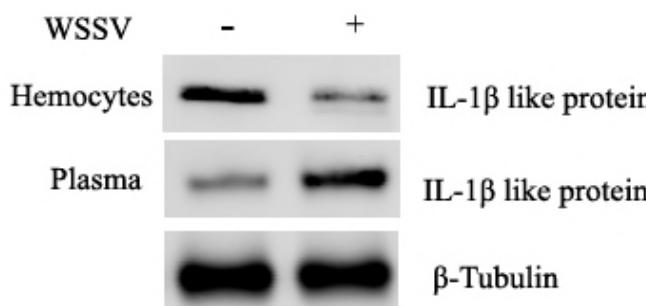


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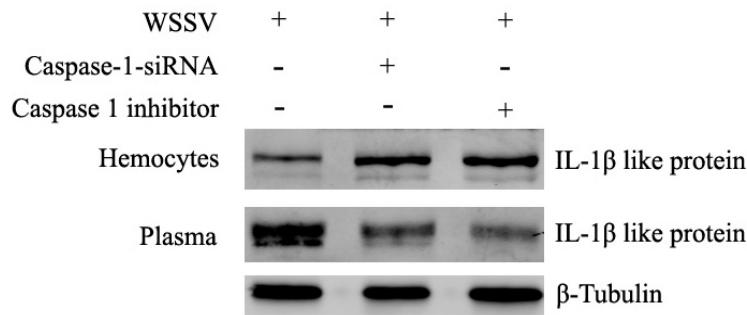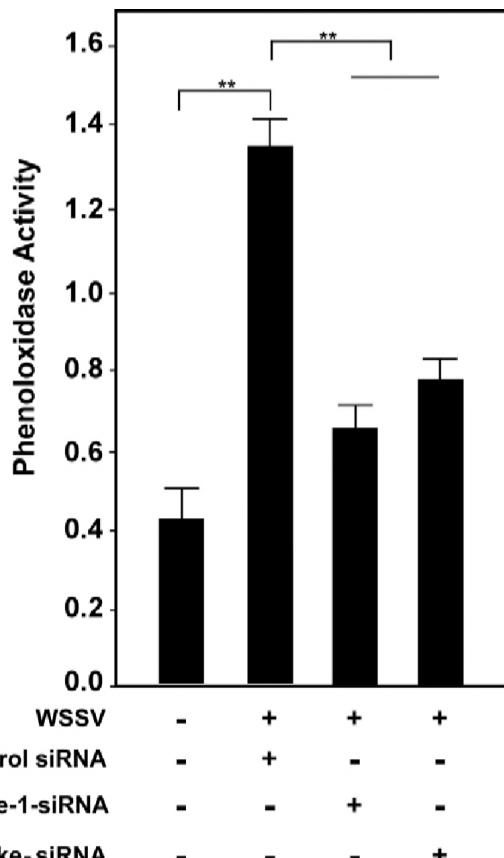
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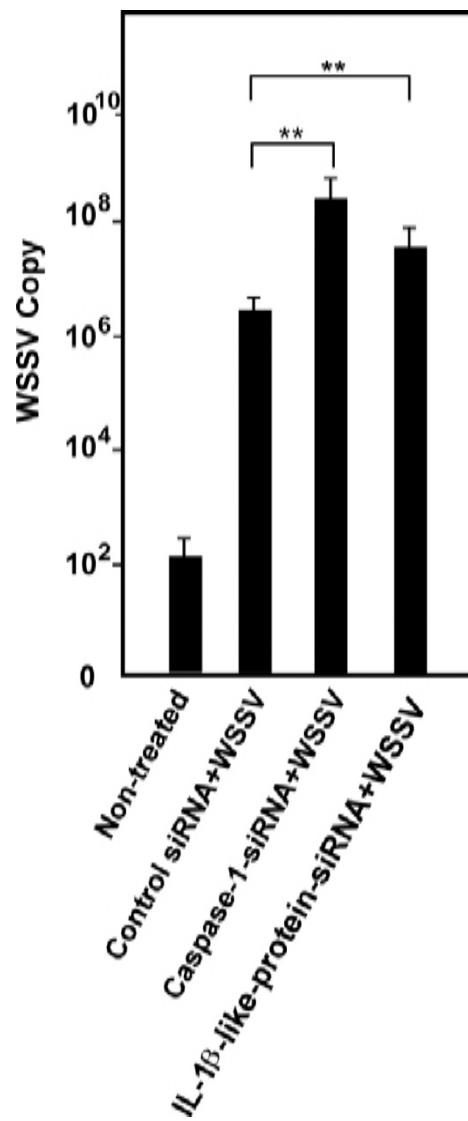


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