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Original article

Peroxiredoxins are important for the regulation of hydrogen peroxide concentrations in ticks and tick cell line

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ABSTRACT

Ticks are obligate hematophagous ectoparasites, as they need to feed blood from vertebrate hosts for development. Host blood contains high levels of iron. Host-derived iron may lead to high levels of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2). Since a high concentration of H_2O_2 causes serious damage to organisms, this molecule is known to be a harmful chemical compound for aerobic organisms. On the other hand, the transparent method is compatible with chemical fluorescent probes. Therefore, we tried to establish the visualizing method for H_2O_2 in unfed tick tissues. The combination method of a chemical fluorescent probe (BES-H_2O_2-Ac) with the transparent method, Sca*l*e, demonstrated in unfed tick tissues that H_2O_2 and paraquat could induce oxidative stress in the tissues, such as the midgut and ovary. In addition, an H_2O_2 detection method using BES-H_2O_2-Ac was established in *Ixodes scapularis* embryo-derived cell line (ISE6) *in vitro* to evaluate the antioxidant activity of peroxiredoxins (PRXs), H_2O_2 scavenging enzymes, against H_2O_2 in the cells. The effects of paraquat in ISE6 cells were also observed in the *PRXs* gene-silenced ISE6 cells. A high intensity of H_2O_2 fluorescence induced by paraquat was observed in the *PRX* gene-knockdowned cells. These results suggest that H_2O_2 and paraquat act as an H_2O_2 inducer, and *PRX* genes are important for the regulation of the H_2O_2 concentration in unfed ticks and ISE6 cells. Therefore, this study contributes to the search for H_2O_2 visualization in ticks and tick cell line and furthers understanding of the tick's oxidative stress induced by H_2O_2.

1. Introduction

Ticks are obligate hematophagous arthropods, as they need to feed on vertebrate blood in all of their developmental stages. Blood feeding provides nutrition and energy for the molting, developing, and vitellogenesis of ticks (Grandjean, 1983). Ticks feed on vertebrate blood that contains high levels of iron, such as heme and ferrous iron (Galay et al., 2015). Host-derived iron may react to oxygen in the tick's body, and then high levels of reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), may be generated (Citelli et al., 2007, Kusakisako et al., 2016b). Since a high concentration of H_2O_2 causes serious damage to membrane lipids, nucleic acid, and proteins (Robinson et al., 2010), this molecule is known to be a harmful chemical compound for aerobic organisms.

In our previous study, a tick peroxiredoxin (PRX), *Haemaphysalis longicornis* 2-Cys PRX (HlPRX2), was identified and characterized (Kusakisako et al., 2016a,b). Knockdown of the *HlPRX2* gene caused significant decreases in the engorged body weight, egg weight, and hatching rate for larvae as compared to the control group (Kusakisako et al., 2016b). In addition, the detection of H_2O_2 after the knockdown of *HlPRX2* and a 1-Cys type of *H. longicornis* PRX (HlPRX, Tsuji et al., 2001) in ticks showed that the concentration of H_2O_2 significantly increased before and after blood feeding. The expression levels of the *HlPRX2* gene in ovaries were higher than in other internal organs, such as the salivary glands, midguts, and fat bodies (Kusakisako et al., 2016b). These results indicate that the regulation of H_2O_2 concentrations in ticks by their PRXs might be important for ovaries to succeed at reproduction.

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To observe the interaction between tick PRXs and H_2O_2 generation in ticks, we established an intracellular H_2O_2 detection method in tick tissues using an intracellular specific H_2O_2-probe (BES-H_2O_2-Ac) and a transparent method, Sca*l*e. The Sca*l*e method is a chemical approach for the fluorescence imaging and reconstruction of transparent tissues of organisms, thus, we considered that the transparent method would be useful for investigating the occurrence or localization of H_2O_2 in tick tissues in tandem with the chemical fluorescent probe. In addition, to evaluate the antioxidant activity of PRXs against H_2O_2 in a tick cell line, the H_2O_2-detection method using a BES-H_2O_2-Ac probe was also established in the tick cell line *in vitro*. We also evaluated the effects on the generation of H_2O_2 in the tick cell line of paraquat which is known to induce oxidative stress in mammalian cells.

2. Materials and methods

2.1. Ticks and animals

The parthenogenetic Okayama strain of *H. longicornis* has been maintained by blood feeding on the ears of Japanese white rabbits (KBT Oriental Co. Ltd, Saga, Japan) in the Laboratory of Infectious Diseases, Joint Faculty of Veterinary Medicine, Kagoshima University (Fujisaki, 1978). Rabbits were cared for in accordance with the guidelines approved by the Animal Care and Use Committee of Kagoshima University (Approval no. VM13007) and maintained under regulated conditions throughout the experiments.

2.2. Ticks exposure to H_2O_2 and paraquat and expression analysis of *HlPRX2* mRNA

To analyze the effects of H_2O_2 against *HlPRX2* gene expression in ticks, the anal pore microinjection (Kariu et al., 2011) of 0.3 µl of 3 or 7% H_2O_2 and 20 mM paraquat (Kumar et al., 2016) to unfed ticks were performed. The Milli-Q was used as a negative control. The 3 or 7% H_2O_2-injected ticks were collected at 0, 15, 30, 60, and 120 min post injection, while the 20 mM paraquat-injected ticks were collected at 0, 12, 24, 48, 72, and 96 h post injection. To extract total RNA, three ticks each time point were homogenized using an Automill (Tokken, Chiba, Japan). The extracted RNA was purified using TRI® Reagent (Molecular Research Center, Cincinnati, OH, USA), and then treated with an RQ1 RNase-Free DNase (Promega, Madison, WI, USA). cDNA synthesis was performed with ReverTra Ace-α-® (Toyobo, Osaka, Japan) following the manufacturer's protocol using 1 µg of total RNA.

The expression analysis of the *HlPRX2* mRNA was performed with real-time PCR using THUNDERBIRD™ SYBR® qPCR Mix (Toyobo) with a 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA). Gene-specific primers were previously described (Kusakisako et al., 2016b). Standard curves were made from four-fold serial dilutions of the cDNA of adult ticks fed for three days. The PCR cycle profile was as follows: initial denaturation at 95 °C for 10 min, 40 cycles of a denaturation step at 95 °C for 15 s, and an annealing/extension step at 60 °C for 60 s. The data was analyzed with 7300 system SDS software (Applied Biosystems). At the first step of real-time PCR, *actin*, *tubulin*, *P0*, and *L23* genes were evaluated for standardization and *L23* was selected as the tick reference in the current study.

2.3. Observation of H_2O_2 fluorescence in tick tissues after H_2O_2 or paraquat injection

To visualize the H_2O_2 occurrence in tick tissues, the intracellular specific fluorescent probe (BES-H_2O_2-Ac, Wako, Osaka, Japan) and the Sca*l*e were adapted to the tick tissues. Unfed ticks were injected with 0.3 µl of 7% H_2O_2 or 20 mM paraquat through the anal pore were dissected at 15 min or 24 h post injection, respectively, and the midgut and ovary were collected. The tissues were incubated with 5 µM BES-H_2O_2-Ac in the tick cell culture medium (mentioned below) without fetal bovine serum (FBS) for 1 h at 34 °C. The tissues were washed with phosphate buffered saline (PBS), and then the Sca*l*e treatment using SCALEVIEW-A2 (Olympus, Tokyo, Japan) was followed according to the manufacturer's protocol. After the Sca*l*e, the tissues were embedded in a Tissue-Tec® O.C.T. Compound (Sakura Finetek, Torrance, CA, USA). Frozen sections from each tissue were cut to a thickness of 10 µm using Kawamoto's film method (Leica Microsystems, Tokyo, Japan) and a cryostat (Leica CM 1850, Leica Microsystems, Wetzlar, Germany). After washing the tissues on the film with PBS, the films were placed on a glass slide and mounted with DAPI (VECTASHIELD®; Vector Laboratories, Burlingame, CA, USA) and then covered with a glass cover. The images were recorded using a confocal laser scanning microscope (LSM700, Carl Zeiss, Jena, Germany).

2.4. Immunostaining of HlPRX2 in tick tissues after H_2O_2 or paraquat injection

To evaluate the effects of H_2O_2 against HlPRX2 in tick tissues, the indirect immunofluorescent antibody test (IFAT) using tick tissues was conducted using the recombinant HlPRX2 specific antisera (Kusakisako et al., 2016b). Unfed ticks injected with 0.3 µl of 7% H_2O_2 or 20 mM paraquat were dissected at 15 min or 24 h post injection, respectively. The midgut and ovary were collected. Dissected organs were fixed in a 4% paraformaldehyde phosphate buffer solution (pH 7.4) at 4 °C overnight. After washing with a sucrose series, organs were embedded in a Tissue-Tec® O.C.T. Compound (Sakura Finetek). Frozen sections from each tissue were cut to a thickness of 10 µm using Kawamoto's film method (Leica Microsystems) and a cryostat (Leica CM 1850, Leica Microsystems). The films were blocked with 5% skim milk in PBS (pH 7.4) (blocking solution) at room temperature (RT) for 1 h and then incubated with a 1:50 dilution of anti-HlPRX2 mouse serum (Kusakisako et al., 2016b) in a blocking solution at 4 °C overnight. After being washed in PBS, the films were incubated at RT for 1 h with a 1:1000 dilution of Alexa Fluor® 594 goat anti-mouse IgG (Invitrogen, Carlsbad, CA, USA) in a blocking solution. After removal of the antibody by washing with PBS, the films were placed on a glass slide and mounted with DAPI (VECTASHIELD®; Vector Laboratories), then covered with a glass cover. The images were recorded using the confocal microscopy (LSM700, Carl Zeiss).

2.5. Culture of cells

The ISE6 cell line from the embryo of *I. scapularis* was grown at 34 °C in L–15 B medium (pH 6.4–6.6) with 10% FBS, 5% tryptose phosphate broth, and antibiotics (Munderloh et al., 1994; Yoshii et al., 2008).

2.6. Detection of H_2O_2 using BES-H_2O_2-Ac in ISE6 cells

ISE6 cells were seeded in a 48-well plate at 300 µl/well of 1.0×10^6 cells/ml and incubated overnight at 34 °C. After removing the supernatants, 500 µl of PBS was added to each well and suspended. After the cells were transferred to a 1.5-ml tube, the cells were centrifuged at 630*g* for 3 min. The supernatants were replaced to 0, 1, 5, and 10 µM BES-H_2O_2-Ac (Wako) in a culture medium without FBS. The cells were incubated at 34 °C for 1 h with 0.1 µM Hoechst 33342 (Dojindo, Kumamoto, Japan) for 30 min. The cells were washed with PBS 2 times, and then 120 µl of the culture medium was added. The cells were transferred to a 96-well black plate at 100 µl/well to measure fluorescence. Fluorescence was detected using a microplate reader (SH-9000Lab, Corona Electric, Ibaraki, Japan) with excitation at 480 nm and emission at 535 nm for the BES-H_2O_2-Ac, and with excitation at 352 nm and emission at 461 nm for the Hoechst 33342.