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Pathogen-Targeted Hydroxyl Radical Generation during Melanization in Insect Hemolymph: EPR Study of a Probable Cytotoxicity Mechanism

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Abstract. The main component of the insect immune system is melanotic encapsulation of pathogenic organisms. Molecular mechanisms of destruction of an encapsulated pathogen are poorly understood. Reactive oxygen species (ROS) are considered as probable cytotoxic agents responsible for destruction of pathogenic organisms in insect hemolymph. In the present work the formation of H_2O_2 during melanization in *Galleria mellonella* hemolymph in the presence of catalase inhibitor NaN_3 was detected. Enhanced rates of H_2O_2 generation were observed in the hemolymph of insects activated by injection of bacterial cells. Using spin trapping technique in combination with electron paramagnetic resonance spectroscopy we demonstrated that production of H_2O_2 in the hemolymph causes the formation of highly toxic reactive oxygen species, hydroxyl radical. However, neither H_2O_2 nor hydroxyl radical were detected in the absence of NaN_3 in agreement with the high catalase activity in the hemolymph. These observations allow us to propose a unique mechanism of pathogen-targeted cytotoxicity based on localized hydroxyl radical generation within a melanotic capsule.

1 Introduction

Melanotic encapsulation of pathogenic organisms is the main component of the insect immune system. During this process a melanotic capsule is formed around a pathogenic organism. The melanization process in insect hemolymph is represented by a cascade of chemical reactions catalyzed by phenoloxidase (PO). An initial stage of this process is the hydroxylation of tyrosine to 3,4-dihydroxy-L-phenylalanine (DOPA) followed by its oxidation to corresponding DOPA-quinone. Subsequent reactions lead to the formation of insoluble polymer melanin [1].

Molecular mechanisms of destruction of an encapsulated pathogen are poorly understood. Reactive oxygen species (ROS) such as semiquinone radicals, superoxide radicals, hydrogen peroxide and hydroxyl radicals are considered as cytotoxic molecules which are responsible for the destruction of pathogenic organisms in insect hemolymph [2, 3]. Earlier we reported the production of potentially cytotoxic DOPA-semiquinone radical during melanization in insect hemolymph [4]. There are also several papers reporting the formation of superoxide radical $O_2^{\bullet -}$ in the hemolymph of insects [5–7]. However, the method employed for superoxide detection in these studies, namely, the nitroblue tetrazolium reduction technique, appears to be controversial [8].

In our previous study we have observed hydrogen peroxide production during oxidation of DOPA by purified PO [9]. The production of H_2O_2 in the DOPA-PO enzymatic system was apparently accompanied by the generation of superoxide radical; however, the $O_2^{\bullet -}$ detection using spin traps was prevented by its effective scavenging by DOPA ($k = 3.4 \cdot 10^5$ $M^{-1}s^{-1}$) [9]. Moreover, the reaction of $O_2^{\bullet -}$ with melanin ($k \sim 10^6$ $M^{-1}s^{-1}$ [10]) may further contribute to its fast decay and low stationary concentration below a detectable level. These facts allowed us to suggest that $O_2^{\bullet -}$ itself plays a limited role in cytotoxic reactions against pathogenic organisms during their encapsulation.

It has been reported that H_2O_2 is produced in the hemolymph during the immune response of *Drosophila melanogaster* [11] and *Anopheles gambiae* [12]. In the present paper we hypothesized that H_2O_2 in insect hemolymph is implicated in cytotoxic reactions against pathogenic organisms via Fenton-like chemistry resulting in the formation of highly reactive hydroxyl radical $OH^{\bullet}$. It is known that insects of various species possess high catalase activity [13]. Catalase can serve for localization of cytotoxic activity of H_2O_2 so that subsequent $OH^{\bullet}$ generation is localized inside the melanotic capsule around the surface of a pathogenic organism. To verify the hypothesis we have studied the production of H_2O_2 during melanization in the hemolymph of *Galleria mellonella* larvae. Using the spin trapping technique we also investigated the possible formation of $OH^{\bullet}$ in the hemolymph in the presence of a catalase inhibitor. The obtained data support the hypothesis that $OH^{\bullet}$ is produced during melanization in insect hemolymph and may essentially contribute to the destruction of pathogenic organisms during their encapsulation.

2 Materials and Methods

2.1 Chemicals

DOPA, catalase from bovine liver, xylenol orange, D-sorbitol, $FeSO_4 \cdot 7H_2O$, NaN_3, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), α-(4-pyridyl-1-oxide)-N-*tert*-butylnitrone (4-POBN), ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma (USA).

All experiments were carried out in phosphate buffered saline (PBS) (50 mM, pH 7.4), containing 150 mM NaCl and 50 μM EDTA.

2.2 Insects and Bacterial Cell

A laboratory population of greater wax moths, *Galleria mellonella* (Lepidoptera: Pyralidae), was reared on artificial diet at 28 °C in the dark. Sixth-instar larvae were used for experiments.

Bacterial cells (*Bacillus thuringiensis galleriae*) were treated with formalin and washed three times with PBS. Suspension of formalin-killed *B. thuringiensis* was used for injections.

2.3 Hemolymph Collection

Hemolymph from *G. mellonella* larvae was collected into 1.5 ml Eppendorf tubes with ice-cold PBS and centrifuged for 5 min at 1000×*g* and 4 °C. Cell-free hemolymph was used in experiments.

2.4 Analysis of H_2O_2 Production in Hemolymph

The ferrous oxidation xylenol orange (FOX) assay [14] was employed to assess the H_2O_2 production in the hemolymph. Measurements were performed in hemolymph samples diluted ten times with PBS. To inhibit H_2O_2 decomposition by endogenous catalase, 1 mM NaN_3 was added to the hemolymph samples. Control samples of hemolymph contained catalase, 200 U/ml, instead of NaN_3. Melanization was initiated by the addition of 1 mM DOPA. To evaluate the rates of H_2O_2 production, the samples were incubated for 30 min and concentration of accumulated H_2O_2 was determined by the FOX assay afterwards. The FOX system was as follows: 312.5 μM $FeSO_4$, 125 μM xylenol orange and 125 mM sorbitol in HCl-NaCl buffer solution (0.3 M, pH 1.4). Mixtures of experimental samples (200 μl) with the FOX system (800 μl) were incubated for 20 min and placed into a quartz cuvette with a diameter of 1 cm for optical measurements. The concentration of H_2O_2 in the sample was determined by monitoring the absorption at 560 nm using a calibration curve obtained with pure H_2O_2. Optical measurements were performed on a UV-2401 (PC) CE spectrophotometer (Shimadzu, Japan).

2.5 Electron Paramagnetic Resonance (EPR) and Spin Trapping

DMPO and 4-POBN spin traps were used to detect ROS production in the hemolymph. Isolated hemolymph was diluted 20 times with PBS and 1 mM NaN_3 was added to inhibit endogenous catalase. For the trapping of secondary radicals produced upon reactions with $OH^{\bullet}$, 2% (v/v) DMSO or ethanol were added to the hemolymph samples. Mixtures of hemolymph and spin trap (50 mM) were placed into glass capillary tubes (volume, 50 μl) for EPR measurements. EPR spectrometer settings were as follows: modulation frequency, 100 kHz; modulation ampli-

tude, 1 G; microwave power, 20 mW. EPR measurements were performed on an ER 200D-SRC X-band EPR spectrometer (Bruker, Germany).

3 Results and Discussion

In our previous work we have shown that the melanization process catalyzed by purified PO is accompanied by the production of H_2O_2 [9]. To elucidate the role of H_2O_2 in insect defense mechanisms we have studied the production of H_2O_2 during melanization in the hemolymph of *G. mellonella* larvae. By the FOX assay we have shown that H_2O_2 is accumulated during melanization in the hemolymph in the presence of a catalase inhibitor, 1 mM NaN_3. However, accumulation of H_2O_2 was not detected in the absence of NaN_3 (data not shown). We have compared the rates of H_2O_2 production in the hemolymph of immunoreactive and control insects. *Galleria mellonella* larvae were injected with 4 µl (10^4 cells per larva) of a suspension of formalin-killed bacterial cells of *B. thuringiensis*. The control group of insects was injected with 4 µl of sterile solution of 150 mM NaCl. 1, 4 and 24 h after the injection, hemolymph was isolated from insects for H_2O_2 analysis. A significantly higher rate of H_2O_2 production was observed in the hemolymph of activated insects 1 and 4 h after the injection (Fig. 1). After 24 h the rates of H_2O_2 production in the hemolymph of both activated and control insects returned to the initial level.

The obtained data demonstrate that the melanization process in insect hemolymph is accompanied by the production of H_2O_2. The increased rate of H_2O_2 production in the hemolymph of activated insects allows us to suggest that H_2O_2 is implicated in cytotoxic reactions against pathogenic organisms during their encapsulation. The toxicity of hydrogen peroxide may be associated with the forma-

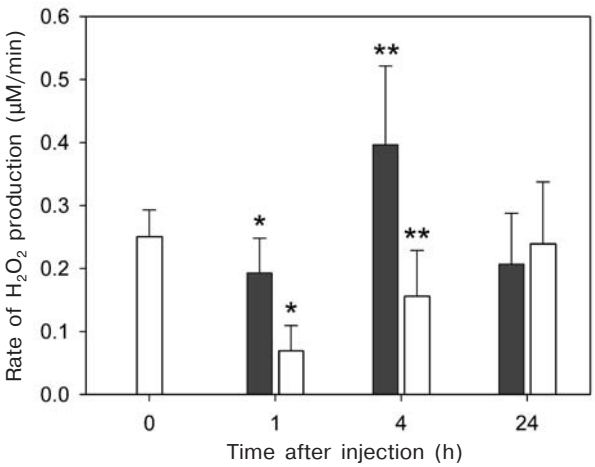

Fig. 1. Rates of H_2O_2 production during melanization in hemolymph from *G. mellonella* larvae activated by injection of formalin-killed bacterial cells of *B. thuringiensis*. White bars, control insects; black bars, activated insects. Data are means with standard deviation (n = 7–20; asterisk, $P < 0.05$; double asterisk, $P < 0.01$).

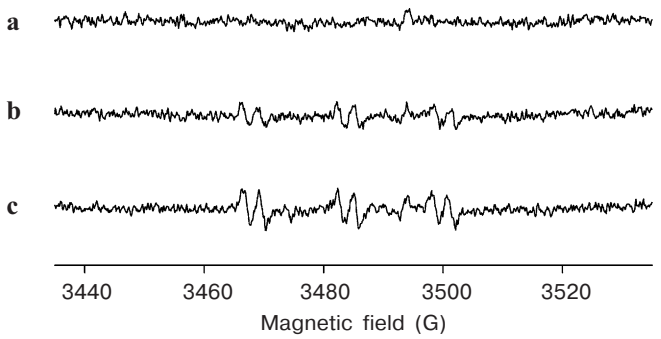

Fig. 2. EPR spectra obtained for *G. mellonella* hemolymph in the presence of catalase inhibitor, 1 mM NaN_3, and spin traps: 50 mM DMPO (**a**); 50 mM 4-POBN and 2% DMSO (**b**); 50 mM 4-POBN and 2% ethanol (**c**).

tion of highly reactive hydroxyl radical via reduction of H_2O_2, e.g., in Fenton-like reactions in the presence of transition-metal ions. To test the possibility of $OH^{\bullet}$ generation in insect hemolymph during melanization we used a spin trapping approach (see Sect. 2). We were not able to detect the adduct of the DMPO spin trap with $OH^{\bullet}$ in the hemolymph (Fig. 2a) probably due to extremely high nonspecific reactivity of hydroxyl radical towards any organic molecules and comparatively short lifetime of the paramagnetic spin adduct. Therefore, we applied another spin trap, 4-POBN, which is a popular choice for EPR detection of second-

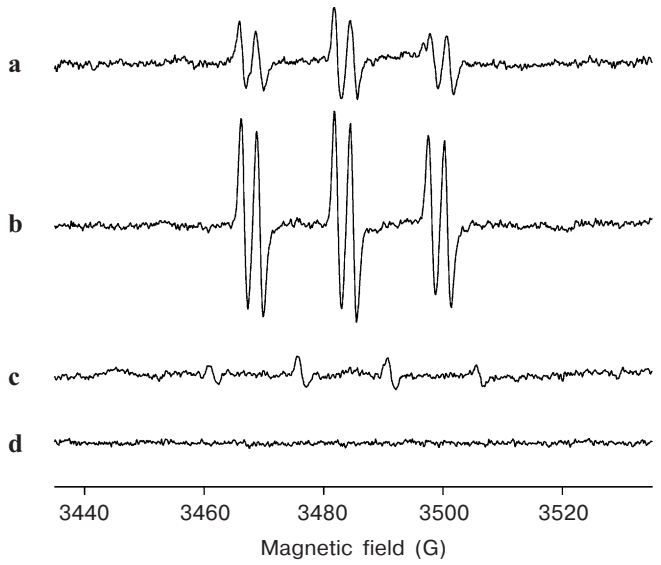

Fig. 3. EPR spectra obtained for *G. mellonella* hemolymph in the presence of catalase inhibitor, 1 mM NaN_3, 5 μM Fe^{2+} and spin traps: 50 mM 4-POBN and 2% DMSO (**a**); 50 mM 4-POBN and 2% ethanol (**b**); 50 mM DMPO (**c**). For spectrum **d**, the spin traps were the same as for **b** but 200 U/ml catalase was used instead of NaN_3.

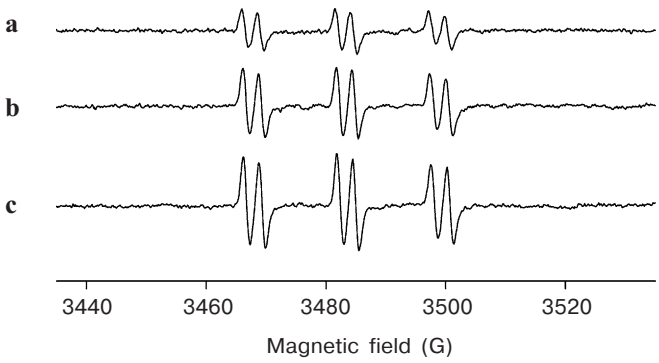

Fig. 4. EPR spectra of *G. mellonella* hemolymph in the presence of 1 mM NaN_3, 5 μM Fe^{2+}, 50 mM 4-POBN and 2% ethanol measured at different time points after addition of the spin trap: 1 min (**a**), 15 min (**b**), and 30 min (**c**).

ary radicals formed upon reactions with $OH^{\bullet}$. For example, EPR detection of comparatively stable spin adducts with methyl radical $CH_3^{\bullet}$ or α-hydroxyethyl radical $^{\bullet}CH(OH)CH_3$ formed upon addition of DMSO and ethanol, respectively, is considered as an unambiguous proof of $OH^{\bullet}$ generation in the systems studied [15]. Figure 2b and c shows the EPR spectra measured in the hemolymph in the presence of catalase inhibitor NaN_3, 4-POBN and DMSO or ethanol, respectively. No EPR spectra were observed in the absence of NaN_3 (data not shown). Significantly more intensive EPR spectra but identical with those shown in Fig. 2b and c were measured in the hemolymph in the presence of NaN_3 and 4-POBN upon addition of DMSO or ethanol in combination with 5 μM Fe^{2+} (Fig. 3a and b). The hyperfine splitting constants of these adducts (a_N = 15.95 G and a_H = 2.70 G, and a_N = 15.70 G and a_H = 2.55 G for 4-POBN in the presence of DMSO or ethanol, respectively) are consistent with the values reported for methyl and α-hydroxyethyl adducts of 4-POBN [16]. The formation of secondary radicals from DMSO or ethanol supports the possibility of $OH^{\bullet}$ generation from the hydrogen peroxide in the hemolymph during melanization. In agreement with this hypothesis we observed a small but identifiable EPR spectrum of DMPO-OH spin adduct in the hemolymph in the presence of 5 μM Fe^{2+} (Fig. 3c). No EPR spectrum was observed in the hemolymph in the presence of 200 U/ml catalase instead of NaN_3 (Fig. 3d). Moreover, the intensity of the EPR spectra of the spin adducts of 4-POBN with α-hydroxyethyl (Fig. 4) and methyl radicals (data not shown) increased with time indicating a continuous production of hydrogen peroxide followed by subsequent hydroxyl radical generation in the hemolymph during melanization.

4 Conclusion

The observed data support the hypothesis that H_2O_2 produced during melanization is involved in cytotoxic reactions against the encapsulated pathogen. This conclusion is also supported by data from Shelby and Popham [17], who have

shown that PO-dependent virucidal activity of the hemolymph from *Heliothis virescens* is inhibited by addition of catalase. The results of the present study also suggest that $OH^{\bullet}$ production contributes to the cytotoxic activity of H_2O_2 in insect hemolymph.

Unlike mammals, insects possess an open circulatory system. Production of reactive species, such as hydrogen peroxide and hydroxyl radical, in these conditions could cause damage of the insect's own tissues. High catalase activity reported for various insects [13] probably serves for effective decomposition of H_2O_2 formed in the hemolymph during the immune response, thereby protecting the insect's organs against oxidative damage. On the other hand, hydroxyl radical reacts with nearby biomolecules with diffusion-controlled rates and, once formed inside the melanotic capsule, it does not have any chance to escape in the outer space. In summary, the enhanced rate of H_2O_2 production followed by subsequent $OH^{\bullet}$ generation during melanotic encapsulation of pathogenic organisms in combination with high catalase activity in insect hemolymph might represent a unique mechanism of local, pathogen-targeted, cytotoxic action.

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