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EPR Detection of Reactive Oxygen Species in Hemolymph of *Galleria mellonella* and *Dendrolimus superans sibiricus* (Lepidoptera) Larvae

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The formation of reactive oxygen species (ROS) in hemolymph and hemocytes of *Galleria mellonella* and *Dendrolimus superans sibiricus* larvae was studied by ESR spectroscopy using spin-trap 1-hydroxy-3-carboxy-pyrrolidine (CP-H). The background level of ROS formation was detected in the intact hemolymph. The addition of dihydroxyphenylalanine (DOPA) into free cells of the hemolymph increased CP-H oxidation about two times for *G. mellonella* and about four times for *D. superans sibiricus*. This increase was completely inhibited by a specific inhibitor of phenoloxidase, phenylthiourea. The presence of exogenous superoxide dismutase (SOD) did not change CP-H oxidation in the hemolymph. The data obtained in hemocytes showed only a DOPA-induced increase in CP-H oxidation. Phagocytosis activators did not affect ROS formation in hemocytes of both insect species. SOD decreased DOPA-induced CP-H oxidation 20–30% in suspension of hemocytes of *D. superans sibiricus* only. Our results are in agreement with the contribution of superoxide radical and DOPA-derived quinones/semiquinones in the immune response of insects.

The encapsulation is a fundamental defense mechanism of insect that prevents the development of pathogen organisms into hemocell. A multilayer cover containing different quinones and melanins forms around pathogens during encapsulation process. The important role in melanogenesis belongs to phenoloxidase (PO) that converts mono- and/or diphenols to quinones which, in turn, polymerize to form melanins [1–3]. The cytotoxic effect of quinones in vertebrates is well known [1, 4]. The quinones may be reduced to semiquinones by the variety of enzyme systems with following interaction with biological macromolecules or with oxygen reducing it to superoxide anion (O_2^{-}). This might result in increased formation of other reactive oxygen species (ROS) including hydrogen peroxide and hydroxyl radical ($OH^{-\bullet}$). ROS can damage different invading foreign microorganisms as well as tissues of the host. However, there are few experimental data concerning the cytotoxicity of quinones and ROS in the insects [5]. The direct registration of ROS in insect hemolymph also was not reported.

In the present work we have studied generation of ROS and other high oxidizing metabolites during melanogenesis in hemolymph of great wax moth (*Galleria mellonella*) and Siberian moth (*Dendrolimus superans sibiricus*) caterpillar by EPR method using spin traps.

MATERIALS AND METHODS

Insects

Larvae of the wax moth *Galleria mellonella* were reared in the laboratory under conditions described in [6]. The larvae of the Siberian moth *Dendrolimus superans sibiricus* were taken from the field (Novosibirsk area). Last-instar larvae of both species of moth were used for the experiments.

Chemicals

1-Hydroxy-3-carboxy-pyrrolidine (CP-H) was kindly provided by Dr. I. Kiriluyk from Novosibirsk Institute of Organic Chemistry. 3(3,4-Dihydroxyphenyl)-DL-alanine (DOPA), phenylthiourea (PTU), deferoxamine mesylate (Df), superoxide dismutase from bovine erythrocyte (SOD), 3-carboxy-proxyl (CP), lipopolysaccharide from *Escherichia coli* K-235 (LPS), zymosan A, phorbol myristate acetate (PMA), spin traps 5,5-diethyl-1-pyrroline-*N*-oxide (DMPO), phenyl *N*-*tert*-butylnitron (PBN) were all purchased from Sigma (U.S.A.). Spin trap 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) was obtained from Oxis International (Portland, U.S.A.). The absence of paramagnetic impurities was checked in all stock solutions of spin traps by EPR spectroscopy. All buffers were passed through a Chelex 100 column to remove divalent metal ion impurities.

Collection of Hemolymph and Hemocytes

The larvae of *G. mellonella* and *D. superans sibiricus* were precooled at −2°C for 5 min and for 15 min respectively, then hemo-

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TABLE 1

ROS Formation in Hemolymph (H) of *D. superans sibiricus* Measured as Rate of Oxidation (nM/s) of Hydroxylamine CP-H to Nitroxyl Radical CP

H	H + PTU	H + DOPA	H + DOPA + SOD
12.4 ± 4.8	2.1 ± 1.0	56.6 ± 12.2*	66.1 ± 15.4**

Note. Data are means ± SD ($n = 14$; *$P < 0.01$ vs hemolymph; **$P > 0.05$ vs hemolymph + DOPA).

lymph was collected from punctured proleg of the larvae into the glass capillary and flushed out into an Eppendorf tube kept on ice. The collected hemolymph was centrifuged at +4–+6°C for 5 min at 500*g*. The resulting hemolymph was diluted 10 times in 50 mM phosphate buffer, pH 7.4, containing 0.15 M NaCl and 50 μM Df (PBS-D) for further use. To isolate hemocytes, hemolymph from 10 larvae of *G. mellonela* and from 3–4 larvae of *D. superans sibiricus* was collected directly into Eppendorf tubes containing 200 μl of ice-cold anticoagulant buffer (AB), consisting of 0.14 M NaCl, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid, pH 4.6 [7]. The hemocytes were washed five times in AB and twice in PBS-D by centrifugation at 500*g* for 5 min, then suspension of hemocytes (5×10^3 cells/μl) in PBS-D was used. The number of hemocytes was counted using a Gorjaev chamber.

Preparation of CP-H Solution

CP-H was dissolved in oxygen-free (argon-bubbled) PBS-D. Deferoxamine was used to decrease the self-oxidation of hydroxylamine catalyzed by traces of transition metal ions. The concentration of CP-H in the stock solution was 10 mM. Before the experiments, stock solution was kept frozen [8].

Purification of DMPO

DMPO was purified by filtration of aqueous solution through charcoal [9]. Elution of DMPO was monitored by its UV absorbance ($\varepsilon_{234} = 7700\ M^{-1}\ cm^{-1}$ [10]) and fractions were checked on the absence of paramagnetic impurities by EPR spectroscopy.

Spin-Trapping Experiments

Superoxide radical detection. Superoxide radical generation in hemolymph and hemocytes was determined using the spin traps DMPO (0.1 M), DEPMPO (0.01 M) and PBN (0.1 M) [11–13]; all samples were prepared in PBS-D (pH 7.4) and were placed in 200 μl flat cell before EPR measurement.

Using hydroxylamine CP-H (1 mM) as spin trap [8]. All samples were prepared in PBS-D (pH 7.4) and were placed in 100 μl glass capillaries of an internal diameter of 1 mm for EPR measurements. Concentrations of DOPA and SOD in mixtures of hemolymph or hemocytes with spin trap were 0.2 mg/ml and 700 U/ml respectively.

EPR measurements were performed at room temperature using an ER 200D-SRC X-band EPR spectrometer (Bruker). The EPR settings were the following: field center, 3474 G; field sweep, 50 G; microwave power, 20 mW; magnetic field modulation, 100 kHz; modulation amplitude, 1G.

Determination of Rates of ROS Formation

CP-H spin trap [8] has been used for the measurement of the rate of ROS formation. CP-H is nonspecifically oxidized by ROS (superoxide, hydroxyl radicals, peroxynitrite and other high oxidizing metabolites) with formation of the stable nitroxyl radical, CP. The observation of time-dependent accumulation of CP radical by EPR was used to follow the kinetics of ROS formation *in vitro* obtained by monitoring the amplitude of the low field component of the EPR spectrum. Experimental concentration of CP *in vitro* was determined using calibration dependence of the EPR amplitude on concentration of CP obtained from Sigma. To clarify the contribution of superoxide radical into the kinetics of CP-H oxidation SOD was used as competitive reagent [8]. We have found that experimental concentration of DOPA (up to 0.2 mg/ml) does not contribute into the rate of CP-H oxidation.

To prove the ability of CP-H to be oxidized by quinone DOPA metabolites we performed the experiment on non-enzymatic oxidation of DOPA (see Fig. 1 and caption).

Statistics

All values are reported as means ± SD. Statistical significance was determined by Student's test for paired data. Two groups of data were considered to be significantly different at $P < 0.05$.

RESULTS AND DISCUSSION

In recent years several investigations have shown that SOD-inhibitable NBT reduction system is detected in hemolymph of different lepidopteran species [14, 15]. These data suggest the presence of source of superoxide radical in hemolymph. The phenoloxidase catalyzes the initial hydroxylation of tyrosine as well as the following oxidation of DOPA. Both processes are the initial reactions of melanogenesis during which the superoxide anion and other ROS may be generated [4, 5].

At present, spin trapping combined with EPR spectroscopy is the most frequently used method for the detection of superoxide anion and hydroxyl radicals. In our work we used widespread nitrone-based spin traps DMPO, PBN and DEPMPO for detection of $O_2^{-\cdot}$ and $OH^{-\cdot}$ radicals in hemolymph and hemocytes in last-instar larvae of *D. superans sibiricus* and *G. mellonella*. However the application of these spin traps did

TABLE 2

ROS Formation in Hemolymph (H) of *G. mellonella* Measured as Rate of Oxidation (nM/s) of Hydroxylamine CP-H to Nitroxyl Radical CP

H	H + PTU	H + SOD	H + DOPA	H + DOPA + SOD
16.0 ± 9.1	3.2 ± 1.2	16.8 ± 10.0***	44.3 ± 16.5*	47.5 ± 16.8**

Note. Data are means ± SD ($n = 14$; *$P < 0.01$ vs hemolymph; **$P > 0.05$ vs hemolymph + DOPA; ***$P > 0.05$ vs hemolymph).

TABLE 3

ROS Formation in Suspensions of Hemocytes (Cells) of *D. superans sibiricus* Measured as Rate of Oxidation (nM/s) of Hydroxylamine CP-H to Nitroxyl Radical CP

Cells	Cells + SOD	Cells + DOPA	Cells + PTU + DOPA	Cells + DOPA + SOD
4.9 ± 2.0	4.8 ± 1.95**	6.4 ± 2.6***	5.0 ± 2.0*	5.3 ± 2.0*

Note. Data are means ± SD ($n = 8$; *$P < 0.05$ vs cells + DOPA; **$P > 0.05$ vs cells; ***$P < 0.05$ vs cells).

not allow us to observe EPR signals of any corresponding spin adducts when phagocytosis activators (LPS, zymosan and PMA) were added to hemolymph and to hemocytes both in the presence and in the absence of DOPA. Taking into account the complicity of the biological systems as well as the low trapping efficiency of the above spin traps, we consider that the absence of EPR signal does not negate the presence of superoxide radical in the insect tissues. Therefore we used more sensitive spin trap CP-H which gives the possibility to quantify the formation of superoxide radical and other reactive oxygen species. The use of this hydroxylamine was shown to increase the sensitivity of superoxide radical detection in about 10 times compared to nitrone-based spin traps [8, 16]. However the parameters of EPR spectrum of spin adduct, CP, are not specific to the type of the ROS.

The rates of ROS generation in hemolymph of wax moth and Siberian moth were assayed as the rates of CP formation and are presented in Tables 1 and 2. It was shown that intact hemolymph of both insect species produces the background level of ROS. The addition of the substrate of phenoloxidase, DOPA, to hemolymph significantly enhanced the rates of ROS generation. SOD did not decrease the rates of CP formation neither in hemolymph per se nor in hemolymph with DOPA. These data indicate that superoxide radical does not significantly contribute to the rates of CP-H oxidation in the free cells hemolymph of larvae in the presence of DOPA or without it. However, we do not exclude the possibility of superoxide formation in these systems. Previously it was demonstrated that hemolymph of different insects, including Lepidoptera, contains SOD [17]. Probably, the inhibitory effect of added SOD on ROS formation was not observed in our experiments because of the high level of endogenous SOD in hemolymph. The rate of CP-H oxidation by intact and DOPA-induced hemolymph was significantly inhibited by the specific inhibitor of phenoloxidase, PTU (see Tables 1 and 2). These data show that DOPA-derived metabolites of phenoloxidase system are mainly responsible for observed oxidation of CP-H in hemolymph.

The rates of CP-H oxidation in the suspension of hemocytes of wax moth and Siberian moth did not differ from CP-H background oxidation in PBS-D (Tables 3 and 4). No effect of phagocytosis activators on the ROS formation in hemocytes was observed both in experiments with nitrone-based spin traps and with CP-H (data not shown). From these results it may be concluded that the generation of superoxide in insect hemocytes does not occur under the action of these activators in contrast to the blood cells of other invertebrate and vertebrate [17]. The rates of CP-H oxidation by hemocytes increased only in the presence of DOPA. PTU and SOD inhibited DOPA-induced CP-H oxidation by hemocytes of *D. superans sibiricus* larvae while SOD had no effect in the case of *G. mellonella* (see Tables 3 and 4).

The obtained data suggest that the oxidation of CP-H to CP in the suspension of hemocytes and free cells hemolymph could occur as the result of interaction of spin trap with ROS. To clarify the types of these ROS the experiments in the presence of catalase and DMSO (scavenger of OH-radical and peroxynitrite [8]) were performed. We have shown that addition of either catalase or DMSO did not affect CP-H oxidation allowing us to exclude hydroxyl radical and peroxynitrite formation in hemolymph and hemocytes of the insects (data not shown). We suppose that observed CP formation in suspension of hemocytes and in hemolymph is the result of the reactions of CP-H with oxidized forms of DOPA and with simultaneously formed superoxide [4, 5] according to the reactions

TABLE 4

ROS Formation in Suspensions of Hemocytes (Cells) of *G. mellonella* Measured as Rate of Oxidation (nM/s) of Hydroxylamine CP-H to Nitroxyl Radical CP

Cells	Cells + SOD	Cells + DOPA	Cells + PTU + DOPA	Cells + DOPA + SOD
2.2 ± 1.1	4.2 ± 0.2	4.15 ± 1.4*	2.3 ± 1.0***	3.9 ± 1.5**

Note. Data are means ± SD ($n = 11$; *$P < 0.05$ vs cells; **$P > 0.05$ vs cells + DOPA; ***$P < 0.05$ vs cells + DOPA).

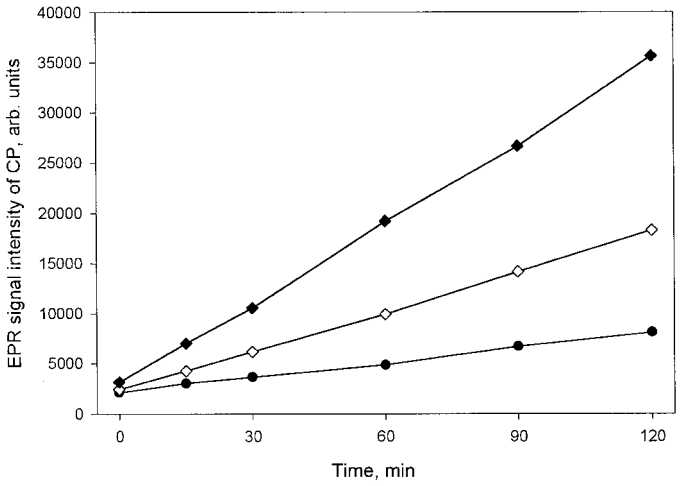

FIG. 1. Time dependence of EPR signal intensity of CP observed in 1 mM solution of CP-H in PBS-D, pH 7.4 in the absence (●) and in the presence (◆, ◇) of 10 mM DOPA. The background formation of CP in the absence of DOPA is due to slow oxidation of CP-H by oxygen. Addition of DOPA resulted in the increase of CP formation (◆). This increase is due to reaction [1] (see Results and Discussion) of CP-H with oxidized forms of DOPA and the reaction [2] with simultaneously formed superoxide as demonstrated by the effect of SOD addition (1000 U/ml) (◇).

$$Q(QH^{\bullet}) + CP - H \rightarrow QH^{\bullet}(QH_2) + CP \qquad [1]$$

$$O_2^{-\bullet} + CP - H \rightarrow CP + H_2O_2, \qquad [2]$$

where QH_2, $QH^{-\bullet}$, and Q are hydroquinone, semiquinone, and quinone of DOPA, respectively.

The oxidation of CP-H by superoxide anion according to the reaction [2] was shown earlier [8, 16]. In the present paper we have shown the possibility of CP-H oxidation in the reaction [1] (see Fig. 1 under Materials and Methods). The absence of inhibitory effect of SOD on CP-H oxidation in hemolymph (see Tables 1 and 2) can be explained by the prevalence of the reaction [1] over the reaction [2] as well as the high level of endogenous SOD in hemolymph.

It is well known that superoxide anion, *o*-quinones and *o*-semiquinones play key role in cytotoxic reactions of vertebrate organisms [18]. Thus, our results support the hypothesis that hemocyte-mediated cytotoxicity in insects may involve different ROS including superoxide and quinoid intermediates of melanin [4, 5]. Bearing in mind the high reactivity of o-quinones/o-semiquinones we consider that these species can be implicated as essential components of the defense mechanism of insect, in particular of *D. superans sibiricus* and *G. mellonella.*

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