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RESEARCH ARTICLE

Dual effect of nitric oxide on phenoloxidase-mediated melanization

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Abstract

The study has demonstrated a dual effect of nitric oxide on phenoloxidase (PO)-mediated DOPA oxidation and melanization process. NO generated at low rates proportionally increased in PO-mediated DOPA oxidation. Competitive PO inhibitor, phenylthiourea, resulted in significant inhibition of NO-mediated DOPA oxidation. Further analysis using fluorescent and EPR methods demonstrated that the effect of NO on DOPA oxidation is explained by oxidation of NO to NO_2 at the active site of PO followed by oxidation of DOPA by NO_2. On the contrary, the bolus addition of NO gas solution resulted in a significant decrease in observed PO activity. Similar dose-dependent effect of NO was observed for the insect's haemocytes quantified as percentage of melanized cells after treatment with nitric oxide. In conclusion, the results of the study suggest that NO may have a significant regulatory role on melanization process in invertebrates as well as in human and result in protective or damaging effects.

Keywords

Melanization, nitric oxide, phenoloxidase

History

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Introduction

Melanization is the process of formation of natural polymer melanin, which is widespread in living organisms. The browning of fruit and root shears, the dark color of animal skin and hair are caused mostly by melanin formation. The process performs various functions in different organisms, the most important of which is the protective activity. Phenoloxidase (PO) is the key enzyme of the melanization process[1,2]. In invertebrates, the melanization underlies the immune response. The penetration of pathogen into an organism initiates the melanization cascade which finally results in the formation of a melanin capsule around the pathogen[3,4]. The particular mechanisms of triggering and regulation of this process were active studying[5], but many aspects are still unclear.

PO (or tyrosinase) refers to oxygenases (monophenol, *ortho*-diphenol: oxygen oxidoreductase, EC 1.14.18.1). It catalyzes the initial steps of the melanization process, namely the hydroxylation of monophenols and oxidation of *ortho*-diphenols by molecular oxygen into the corresponding *ortho*-quinones. Then these quinones undergo a spontaneous intramolecular cyclization to yield indoles that polymerize subsequently into melanin through a cascade of reactions. The active site of PO contains a coupled binuclear copper similar to the active site of haemocyanin, the arthropod's oxygen carrier protein. Phenoloxidases are widely distributed in living organisms: from bacteria to mammals. Human and mouse phenoloxidases are the transmembrane enzymes consisting of two subunits, whereas those of fungi and plants have only one not membrane-bound subunit[1,2,6,7]. The phenoloxidases selected from the haemolymph of certain insects are very similar to that of fungi[8,9]. On substrate oxidation, the active site of PO turns step-by-step into three states: *met*- with Cu(II)–Cu(II), *deoxy*- Cu(I)–Cu(I), and *oxy*- Cu(II)–O_2–Cu(II)[7,10,11]. The native PO is presented mainly by the *met*- form. Some investigations[11,12] report that NO manifests a high affinity for copper-containing enzymes and proteins, such as hemocyanin, tyrosinase, laccase, and cytochrome c.

In the case of humans, melanin pigmentation is stimulated mainly by ultraviolet radiation (UVR). The chemical NO donors are shown to mimic the melanogenic effects of UVR by increasing the PO activity and, hereby, the melanin synthesis in human melanocytes. In author's opinion[13,14], increasing in melanin formation in human melanocytes by NO is achieved rather through cGMP path than by increasing activity of phenoloxidase due to the interaction of PO with NO.

Another effect of nitric oxide on the mushroom PO-mediated oxidation of dopamine and DOPA is reported by Nappi and Vass[15]. NO was found to promote a significant decrease in the total amount of DOPA and dopamine oxidized under experimental conditions. The reported inhibitory effect of NO on the PO activity was assumed to result from the formation of a copper–nitrosyl complex at the active site of enzyme. These findings allow suggesting that NO may have a dual effect on melanization and thereby may have regulatory role in melanization process in invertebrates as well as in human and result in protective or damaging effects. The aim of our work was to understand how

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interaction of NO with PO affects the enzyme activity and melanization.

Materials and methods

Chemicals

3-(3,4-Dihydroxyphenyl)-L-alanine (DOPA), mushroom phenoloxidase (PO), and phenylthiourea (PTU) were purchased from Sigma (St. Louis, MO). Nitronylnitroxyl radical (4-methoxy-5,5-dimethyl-2-(pyridine-3-yl)-4,5-dihydroimidazol-1-oxyl), NNR, was kindly provided by Dr. I. Kirilyuk (Institute of Organic Chemistry of RAS SB, Novosibirsk, Russia). Fluorescent probe DAF-FM (4-amino-5-methylamino-2',7'-difluorofluoresceine) was purchased from Molecular Probes (Eugene, OR).

Synthetic donor, PAPA NONOate (PAPA/NO, (3-(2-hydroxy-2-nitroso-1-propylhydrazino)-1-propanamine), purchased from Cayman (Ann Arbor, MI) was used as a compound to generate NO. PAPA/NO decomposes to release two NO molecules into solution with a half-life of 77 min at neutral pH and room temperature[16]. Stock solution was prepared in 10 mM NaOH and stored at −20 °C. The concentration was determined from absorbance values at 250 nm in 10 mM NaOH ($\varepsilon = 8$ $M^{-1}\,cm^{-1}$). The rate of NO generation was determined in diluted PAPA/NO solution by EPR method using NNR as NO-scavenger[17]. An aliquot of PAPA/NO stock solution was diluted with phosphate buffer to 0.5 mM and 0.1 mM NNR was added. NO formation rate was determined from decrease of EPR signal of NNR taking into account that stoichiometry of the reaction between NNR and NO is 1:1. The required rate of NO generation was regulated by appropriate dilution of the PAPA/NO stock solution.

Measurements were performed using the Bruker X-band EPR spectrometer ER 200D-SRC (Bruker Corporation, Billerica, MA). Gaseous nitric oxide was prepared by reduction of $NaNO_2$ with $FeSO_4$ at the acidic conditions and purified from higher oxides by passing through a deaerated NaOH solution. Assays were performed using NO-saturated solution, prepared by bubbling NO gas in deaerated PBS. The concentration of NO in stock solution was assumed as 2 mM[18].

Monitoring of DOPA oxidation by phenoloxidase

The experimental mixtures contained 20 U/ml PO and the various concentrations of DOPA. To determine the NO effect, an appropriate concentration of either PAPA/NO or NO gas solution was added to the mixture before the addition of DOPA. In certain experiments, 0.5 μM PTU was added to a solution of PO. The sample was placed into a 1 cm quartz cell and the rate of DOPA-chrome formation was measured at 490 nm ($\varepsilon = 3700$ $M^{-1}\,cm^{-1}$)[19] using an UV-2401 (PC) CE (''Shimadzu'' Corp., Tokyo, Japan). Experiments were carried out at 28 °C.

Detection of DAF-FM triazole formation

To test the interaction between PO and NO, the time course of fluorescent DAF-FM triazole intensity dependent on PO concentration was studied using a DAF-FM probe[20]. Experimental mixtures contained 5 μM DAF-FM, PAPA/NO (NO generation rate ~ 2 μM/min), and PO 200, 600, or 1200 U/ml in PBS. Fluorescence kinetics were recorded using a microplate fluorometer. Experimental conditions are described in[21].

Detection of half-met form of phenoloxidase by EPR

The mixture of 0.5 mM PO and 10 mM PAPA/NO in PBS was incubated for 30 min at ambient temperature. Then, glycerol was added to the experimental sample up to 50% (v/v) and the EPR spectrum was recorded at 105 °K using an Elexsys E 560-P spectrometer (Bruker Corporation, Billerica, MA).

Detection of melanization in Galleria mellonella haemocytes

Preparation of haemocyte samples from haemolymph of *G. mellonella* larvae was carried out according to[22]. Haemocyte suspension (10^4 cells) was placed on a microscope slide. The slides were kept in a moist chamber at 22 °C for 15 min to allow haemocytes to adhere and spread. To fix the haemocytes on the slides, acetone (100 μl) was added and the slides were kept in a moist chamber in the dark for 10 min. Then the haemocytes on the slides were washed three times in PBS, and 20 mM DOPA was added to all the samples and either PAPA/NO (30 μM/min) or the buffered solution of NO gas (25 μM) was added to the experimental slides. The slides were stored in the dark moist chamber for 40 min and then washed with distilled water and checked for the presence of dark-grey deposits in haemocytes (indicative of melanin formation) using a light microscope LUMAM R3 (Russia). The percentage of melanized haemocytes was calculated.

All experiments were carried out in the phosphate buffer solution (PBS, 50 mM Na–phosphate, 150 mM NaCl, pH 7.4) prepared with bidistilled deionized water.

Statistical analysis

The data were analyzed using the software SigmaPlot for Windows, version 9.0 (Systat Software, Inc., San Jose, CA). When necessary, a statistical analysis was performed and the data were expressed as means ± SE ($n \geq 5$). Significant differences of data were analyzed by Student's *t*-test ($p < 0.05$).

Results and discussion

The effect of nitric oxide from PAPA/NO on DOPA oxidation catalyzed by PO

To evaluate the NO effect on DOPA oxidation, catalyzed by phenoloxidase, the experiments were carried out at varying concentration of PAPA/NO. Preliminarily, a mixture of PO and PAPA/NO was incubated for 2 min to reach stationary condition of NO generation. Thereafter 1 mM DOPA was added and the kinetics of DOPA-chrome formation, the product of DOPA oxidation, was registered with a spectrophotometer. Figure 1 demonstrates the kinetics of DOPA-chrome formation at different PAPA/NO concentrations. As a result, the nitric oxide activates the DOPA oxidation depending on PAPA/NO concentration. Figure 2 shows the calculated rates of PO-mediated DOPA-chrome formation versus the NO generation rates (close circles). For comparison, the oxidation of DOPA in the presence of PAPA/NO without enzyme was also registered (Figure 2, open circles). In both cases, the increase of NO generation rate results in a proportional increase of the DOPA oxidation rate. However, in the presence of PO, it was much more effective (slope 1.1 ± 0.6) than for the non-enzymatic pathway (slope $0.0\ 25 \pm 0.0008$). It seems that the nitric oxide causes the increase in the apparent PO activity.

To evaluate the observed effect of NO on PO activity, the dependence of the initial rate of PO-mediated DOPA oxidation on DOPA concentration was investigated. Figure 3 compares this dependence with control samples (without PAPA/NO). The data of fitting in terms of the Michaelis–Menten equation demonstrate that V_{max} increases from 18 ± 0.8 μM/min to 23 ± 1.5 μM/min, whereas the K_M value remains almost the same (0.30 ± 0.04 mM for control samples and 0.24 ± 0.05 mM with PAPA/NO). These data allow the conclusion that NO generated from PAPA/NO has

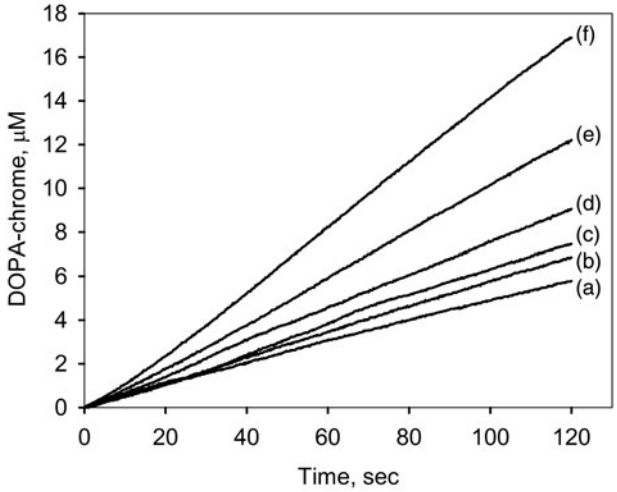


Figure 1. The time curves of DOPA-chrome formation during oxidation of DOPA (1 mM) by phenoloxidase (20 units/ml) in the presence of PAPA/NO: (a) 0 mM, (b) 0.05 mM, (c) 0.1 mM, (d) 0.25 mM, (e) 0.5 mM, (f) 1 mM. $T = 28\,^{\circ}C$, $\lambda = 490$ nm.

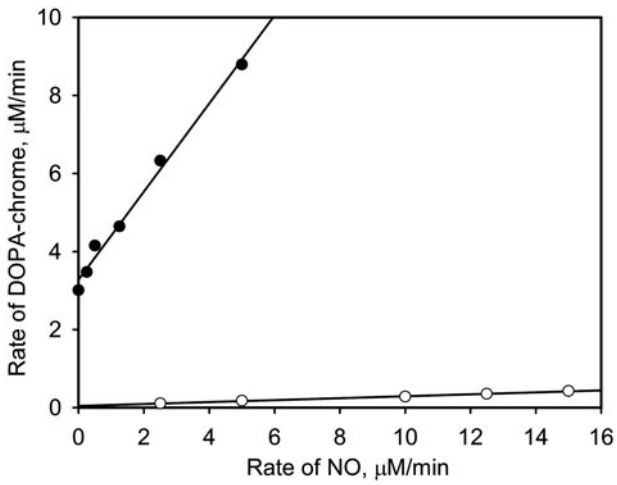


Figure 2. The initial rates of DOPA-chrome formation versus the NO generation rates in the presence of PO (close circles) and without (open circles). The data were obtained by spectrophotometric method under conditions given in Figure 1.

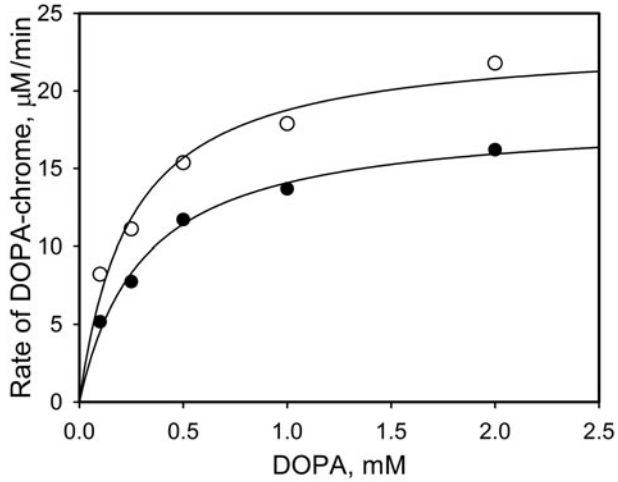


Figure 3. The initial rates of PO-mediated DOPA-chrome formation versus DOPA concentration in the presence of PAPA/NO (0.4 mM, open circles) and without (close circles). The concentration of PO in mixtures was 20 units/ml.

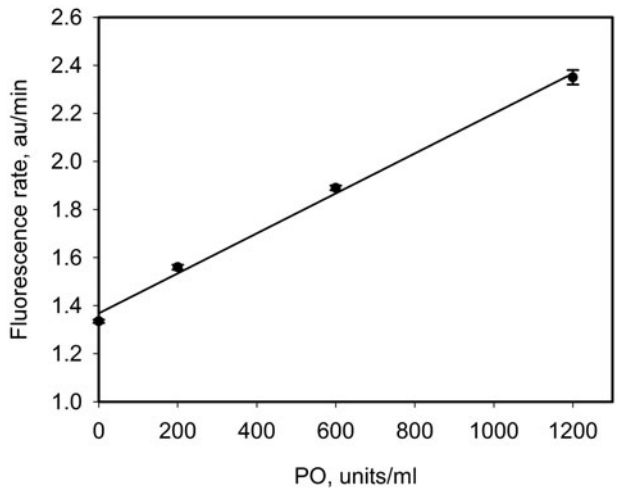


Figure 4. Dependence of the rate of DAF-FM-triazole formation on PO concentration in the mixture of PAPA/NO (NO generation rate of 2 μM/min) and DAF-FM (5 μM).

no essential effect on the PO activity, but leads to the additive PO-mediated pathway of DOPA oxidation.

The nitric oxide can react with the *met-* form of PO (Cu^{2+}–Cu^{2+}) reducing it to the *half-met* PO (Cu^{2+}–Cu^{1+})[23–25]. It is reasonable to propose that the nitric oxide is oxidized hereby to the highly reactive species, namely nitric dioxide, NO_2. Thereafter, it could oxidize DOPA to DOPA-chrome. The next chemical reactions can be involved in this process:

$$2NO + O_2 \rightarrow 2NO_2^{\bullet} \quad (1)$$

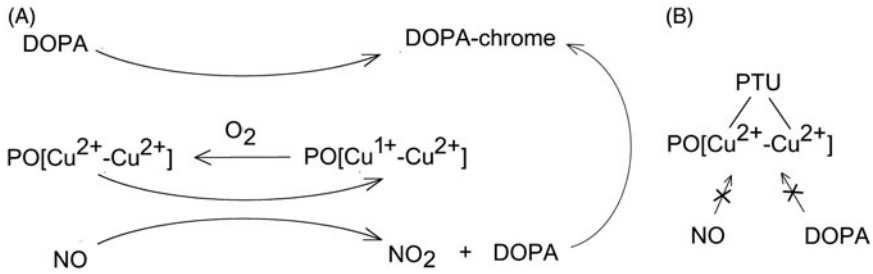
$$PO^{met} + NO \xrightarrow{O2} PO^{half-met} + NO_2^{\bullet} \quad (2)$$

$$NO_2^{\bullet} + DOPA \rightarrow DOPA - chrome \quad (3)$$

Reaction (1) demonstrates that NO_2 formation in aqueous aerobic solutions is of second order in NO. At low NO generation rate (as for PAPA/NO), this reaction could be very slow[26]. In our experiments, this condition was realized in the system without PO which results in a slight oxidation of DOPA (Figure 2, open circles). Reactions (2) and (3) describe the processes of PO-mediated oxidation of NO to NO_2, followed by the oxidation of DOPA to DOPA-chrome. It is assumed, then, that the observed effect of PAPA/NO on the PO-mediated oxidation of DOPA (Figures 1 and 2) is due to the DOPA oxidation by NO_2 in addition to its enzymatic oxidation. NO_2 can be formed by NO oxidation at the active site of PO.

Nitric oxide oxidation in the presence of PO

To study PO-mediated oxidation of NO to NO_2 proposed above, we apply the fluorescent method using DAF-FM as a probe. This fluorescent probe is widely used to register nitric oxide; however, it does not react directly with nitric oxide but with its oxidized forms, NO_2[27] or N_2O_3[20], resulting in the formation of a highly fluorescent product, DAF-FM triazole. In our previous study[21], we have observed that the rate of DAF-FM triazole formation is proportional to generation rate of NO_2. In the present study, we added PO to the system PAPA/NO and DAF-FM. According to Reactions (1) and (2), the formation of NO_2 followed by DAF-FM triazole production should depend on PO concentration. Figure 4 demonstrates the obtained linear dependence of fluorescence rate



Scheme 1. (A) Simplified scheme of DOPA-chrome formation by enzymatic path and NO-mediated proposed path. (B) Inhibition of NO-mediated DOPA-chrome formation by PTU.

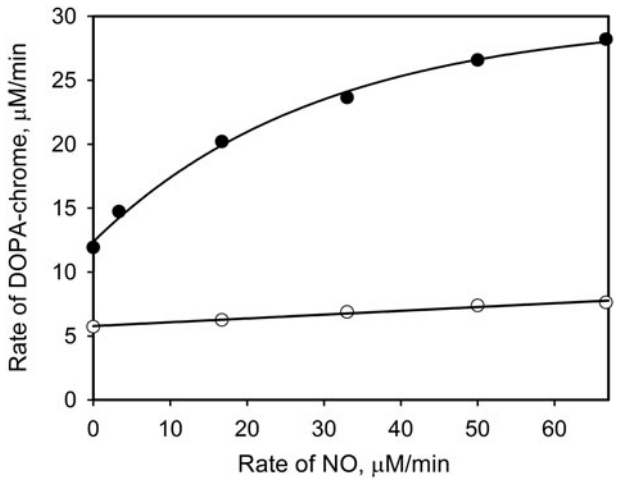


Figure 5. The initial rates of DOPA-chrome formation versus the NO generation rates in the presence of PTU (0.5 μM, open circles) and without (close circles). Reaction mixtures contained 1 mM DOPA, 10 units/ml PO and varying concentrations of PAPA/NO.

versus PO concentration. This result indicates that the formation of NO_2 from NO depends on the presence of PO.

Therefore, the finding support the above statement that the apparent increasing effect of PAPA/NO on the rate of PO-mediated DOPA oxidation could be explained by the additional pathway of oxidation of DOPA by NO_2 formed in this system. Scheme 1(A) describes two proposed paths of DOPA-chrome formation: enzymatic and NO-mediated. Oxidation of NO in this system is presented as the enzymatic-like process, where the active center of PO undergoes re-oxidation by oxygen.

The effect of nitric oxide on PO-mediated DOPA oxidation in the presence of inhibitor

As was proposed above, oxidation of nitric oxide to dioxide can occur at the active site of PO. Under this assumption, the addition of a competitive inhibitor of PO, notably PTU[28,29], should suppress PO-mediated oxidation of NO. It is based on the reported data that PTU bounds to both the copper ions at the active site of catechol-oxidase[30]. The mixtures of PO, PTU, and the different concentrations of PAPA/NO were incubated for 2 min. After that 1 mM DOPA was added and the time-dependent accumulation of DOPA-chrome was measured. Figure 5 demonstrates the dependences of the rate of PO-mediated DOPA-chrome formation versus the rate of NO generation by PAPA/NO in the presence of PTU (open circles) and without (close circles). Increasing effect of NO on the DOPA oxidation rate in the presence of the inhibited PO is negligible, which confirms the supposition that NO is oxidized at the active site of PO.

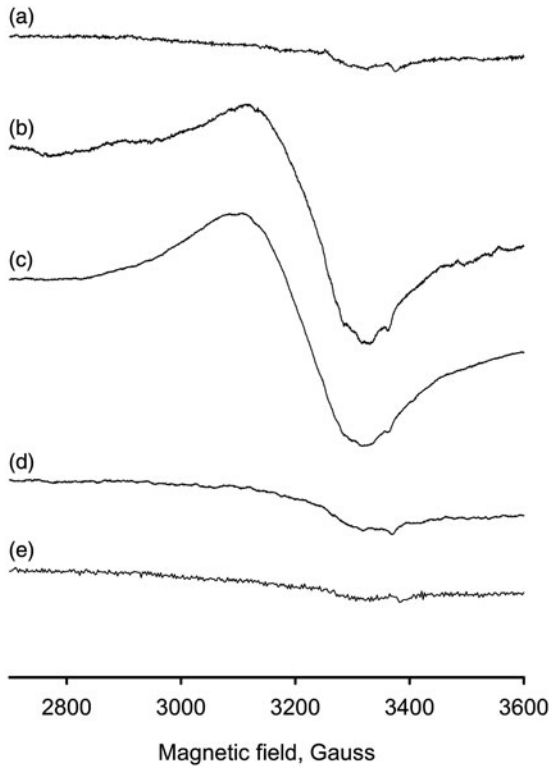


Figure 6. EPR spectra of (a) native phenoloxidase (0.5 mM), (b) phenoloxidase (0.5 mM) incubated for 30 min with 10 mM PAPA/NO, (c) mixture b with 0.5 mM PTU added after incubation with PAPA/NO, (d) mixture of phenoloxidase (0.5 mM) and PTU (0.5 mM) incubated for 30 min with 10 mM PAPA/NO, (e) native phenoloxidase (0.5 mM) with 0.5 mM PTU. All samples were prepared in PBS buffer (pH 7.4) with 50% glycerol. $T = 105\,^{\circ}K$.

Interaction between nitric oxide and the active site of PO observed by EPR method

The *met-* form of PO, which is the main for native enzyme, contains two Cu^{2+} ions at the active site[7]. The EPR spectrum of PO is, however, unobservable due to strong antiferromagnetic interactions between ions[23]. Nitric oxide is known to convert the *met-* into the *half-met* form of PO due to reduction of one copper ion that can be detected by EPR method[23]. It was revealed the final form of enzyme contains the *half-met*-centre [Cu^{1+}–Cu^{2+}] without NO bound[31]. We performed the EPR measurements of PO at the temperature of liquid nitrogen (Figure 6). The incubation of PO with PAPA/NO resulted in the appearance of EPR spectrum. Adding the competitive inhibitor, PTU, to this sample had no effect on the spectrum. Nevertheless, the incubation of PO in reverse order, i.e. first with PTU and then with PAPA/NO, failed to give the EPR spectrum. This observation pointed that PTU

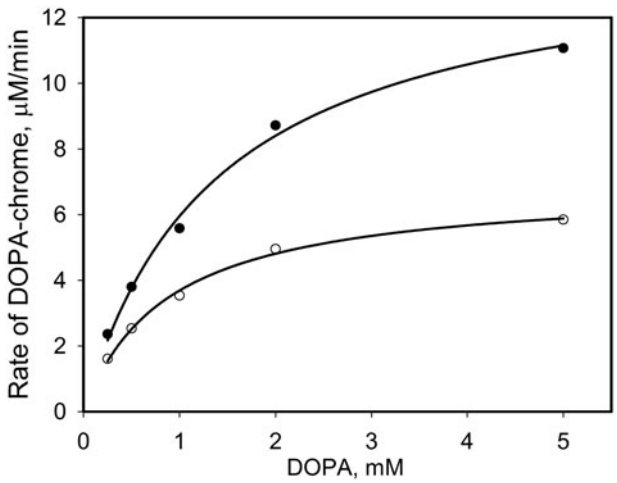


Figure 7. The initial rates of PO-mediated DOPA-chrome formation versus DOPA concentration with bolus addition of NO (25 μM, open circles) and without NO (close circles). The concentration of PO in mixtures was 20 units/ml.

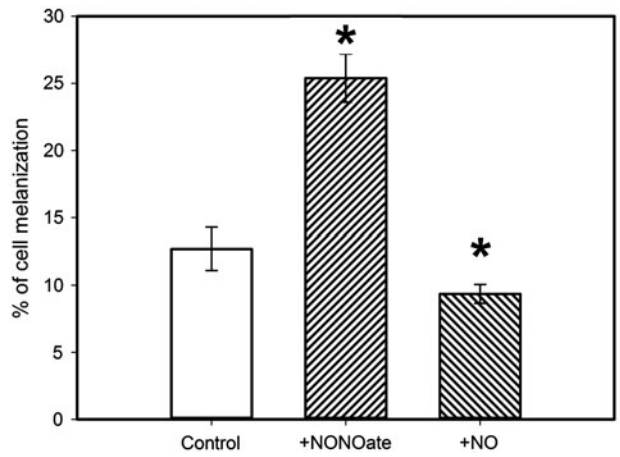


Figure 8. The quantity of melanized haemocytes in samples after incubation with PAPA/NO (NO generation rate of 30 μM/min) and bolus addition of NO (25 μM). *$p \leq 0.05$ in comparison with the control samples.

blocked NO interaction with PO active site by binding both Cu-ions. No spectra were registered in the control samples with PO alone and in the PO/PTU mixture. EPR results are in fair agreement with those presented in Figure 5 obtained by the spectrophotometric method. These findings together indicate the interaction and redox transformations between NO and binuclear copper active center of PO (Scheme 1A and B).

The effect of one-time high-dose treatment of PO by nitric oxide on DOPA oxidation

To test the effect of high local NO concentration on PO activity, we used gaseous nitric oxide solution. The aliquot of the NO-saturated solution was added once up to 25 μM to a PO solution in PBS. After 5 mins' incubation, DOPA was added to the mixture and the kinetics of DOPA-chrome formation was recorded. Figure 7 shows the effect of bolus addition of NO on the rates of PO-mediated DOPA oxidation at different DOPA concentrations. A significant decrease of apparent PO activity was observed. This effect of NO can be assigned to the inhibition of PO via the oxidative damage of enzyme. The main candidates, which can aggressively attack the PO, are the nitric dioxide and peroxynitrite ($ONOO^-$) at high concentrations. The first one is formed due to the oxidation of NO (Reactions 1 and 2), and $ONOO^-$ is the product of the reaction between nitric oxide and superoxide formed during the DOPA oxidation[32]. An inhibitory effect of NO on DOPA oxidation by DEA NONOate at the high NO generation rate was also reported[15]. However, the authors proposed another mechanism of PO inhibition. They suggested that the nitrite derived from NO can interact with the *met*-form to yield a non-catalytic *half-met*-form of the enzyme, or NO directly interacts with *met*-form to produce a *met-nitrite* form, which is also non-catalytic.

The effect of nitric oxide on melanization process in haemocytes

The PO-mediated DOPA-chrome formation is the first stage of the melanization process that occurs particularly in the insect's haemolymph in immune response. The PO is found in the immunocompetent cells of insects, haemocytes. PO catalyses the melanization of haemocytes attached to the surface of a parasite[33].

We have tested the effect of nitric oxide on the melanization of the haemocytes of *G. mellonella* larvae. Experiments were carried out using the haemocytes isolated and fixed on slides. Figure 8 demonstrates the changes in the quantity of melanized cells in the samples treated with PAPA/NO (NO generation rate ~ 33 μM/min) and with NO-gas solution (25 μM) in comparison with the control samples. In the case of PAPA/NO treatment, the quantity of haemocytes with melanization increased, whereas NO-gas solution inhibited melanization in haemocytes. Thus, the result obtained with insect haemocytes corresponds to our data obtained for the model system of mushroom PO.

Conclusions

The present investigation has demonstrated the dual effect of nitric oxide on the PO-mediated DOPA oxidation process. DOPA-chrome formation increased during continuous low-rate generation of NO. The mechanism of this effect involves oxidation of nitric oxide to dioxide at the active site of PO, which thereafter oxidizes DOPA to DOPA-chrome. On the contrary, a significant decrease of DOPA-chrome formation was observed in the case of bolus addition of NO solution to PO due to the inhibition of PO activity.

A similar, dual dose-dependent effect of NO on the melanization process was demonstrated for isolated haemocytes of *G. mellonella* larvae. It is reasonable to assume that these effects of NO have place *in vivo*. We suppose that enhancement of melanization by NO may occur upon encapsulation during immune response in haemolymph of insects. However, it has to be noted that NO-mediated acceleration of melanization may be achieved in the case of NO generation rate comparable with native PO-dependent DOPA oxidation rate. The inhibitory effect of NO requires an enormous local concentration of nitric oxide. This mechanism can be realized in the case of local burst of NO generation or intensive exogenous treatment with nitric oxide. Furthermore we suggest that inhibitory effect of NO on melanization process may protect from harmful action under severe conditions in which the overproduction of melanin results in human diseases.

Acknowledgements

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Declaration of interest

The authors report that they have no conflicts of interests. The work was supported by the Federal Fundamental Scientific Research Programme for 2013–2020 (VI.51.1.5 and V.44.1.3).

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