Effects of Diethylthiocarbamate and Sodium Thiosulfate on the Detection of Lipoprotein Hydroperoxide

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The effects of insect haemolymph melanization inhibitors, diethylthiocarbamate (DDC) and sodium thiosulfate, on the detection of lipoprotein hydroperoxide were examined. The hydroperoxide group (36 μM) of lipoprotein disappeared completely after 2 h incubation with 1 mM DDC. In the presence of 20 mM sodium thiosulfate, only a portion of the lipoprotein hydroperoxide (LDL-OOH) was consumed in 24 h incubation. The hydroperoxide of lipoprotein became sensitive to sodium thiosulfate in the presence of lipoprotein lipase, a digestive enzyme of lipoprotein. In an incubation of lipoprotein with lipoprotein lipase, sodium deoxycholate and sodium thiosulfate, the hydroperoxide content decreased rapidly. The hydroperoxide group in cumene hydroperoxide was degraded in an incubation with sodium thiosulfate, as was the case for the enzyme-digested LDL-OOH. From the data, an accessibility of the reductant to the hydroperoxide group was assumed to affect the degradation of the hydroperoxide. Based on these results, it was suggested that the lipoprotein hydroperoxide could be detected in the presence of sodium thiosulfate, but not with DDC.

Key words: haemolymph, hydroperoxide, melanization inhibitor, diethylthiocarbamate, sodium thiosulfate

INTRODUCTION

Reactive oxygen species participate in various physiological events such as immunological defense or oxidative stresses in vivo. In recent years, oxidative stresses in insects caused by various origins such as plant chemicals have attracted increasing attention (Summers and Felton, 1994). The detection of oxidatively damaged substances in vivo provides indirect evidence of such stress. A superoxide productive system was demonstrated to be present in insect haemolymph plasma (Arakawa, 1994). A detection of oxidized products such as hydroperoxide in the haemolymph is necessary to analyse the oxidative stresses potentially caused by the superoxide production.

Insect haemolymph melanises rapidly after bleeding due to an intrinsic enzymatic cascade, the prophenoloxidase activating system. Various inhibitors are used to block the haemolymph melanization. Phenylthiourea (PTU) is commonly used to inhibit the reaction. However, PTU is unsuitable for the study of oxidative stresses because the chemical interferes with the detection of the hydroperoxide group in plasma lipoprotein (Arakawa, 1995). The effect of PTU is not a masking of the hydroperoxide from a detective probe but a degradation of the hydroperoxide. Diethylthiocarbamate (DDC) and sodium thiosulfate are also used as melanization inhibitors. DDC is an inhibitor of phenoloxidase (Nakamura and Sho, 1964), and sodium thiosulfate is a reductant preventing the oxidation of phenolic compounds (Terasaki et al., 1990). In the present study, the effects of DDC and sodium thiosulfate on the detection of hydroperoxide were examined.
MATERIALS AND METHODS

Insects. Larvae of the rice armyworm, *Pseudaelia separata*, were reared on an artificial diet “INSECTA LF” (Nihon Nohsan Kogyo) under long-day photoperiodic conditions (16L:8D) at 25°C.

**Inhibition of melanization in haemolymph plasma.** The effective dose of sodium thiosulfate for inhibition of haemolymph melanization was determined. Sodium thiosulfate was added to a phosphate-buffered saline (PBS, 50 mm, pH = 7.0) at 0, 20, 40, 100 or 200 mm. Haemolymph was collected from 30 specimens of 4-day old 6th-stadium *P. separata* larvae, and combined in an ice-cold dish (final volume 4.4 ml). Cell-free plasma was obtained by centrifugation at 2,000×g for 3 min. Aliquots from the plasma (500 µl each) were mixed with the same volume of the above PBS containing sodium thiosulfate, which resulted in final inhibitor concentrations of 0, 10, 20, 50 and 100 mm. PBS containing 0.2% PTU was similarly used. These mixtures were incubated at 25°C under darkness in 1.5 ml centrifuge tubes. The melanization was examined at 30 min and 6 h incubation. The extent of blackening and precipitate formation were observed by eye. In addition, dopachrome formation was assessed by absorbance at 490 nm. Distilled water served as a blank. The effective dose of DDC was also examined. DDC was adjusted to the final concentration of 0.05, 0.1, 0.5 and 1 mm in the diluted haemolymph plasma. Mixtures were incubated as above. The melanization was observed by eye.

**Detection of lipoprotein hydroperoxide in the presence of melanization inhibitors.** Human plasma low density lipoprotein (LDL, 5 mg/ml, Sigma) served as a model lipoprotein. Experiments were performed with aliquots from the same bottle of LDL solution. Fifteen microliters of the LDL solution was mixed with 485 µl of PBS (0.1 m, pH = 7.0) containing 1 mm ethylenediamine tetraacetic acid (EDTA) and 0, 5, 20 or 50 mm sodium thiosulfate. PBS containing 1 mm EDTA and 0.05, 0.1 or 1 mm DDC was also similarly used. These mixtures (500 µl each) were incubated at 25°C under darkness and their hydroperoxide content was monitored by the methyleneblue-haemoglobin method (MB-HG method, Ohishi et al., 1985) using an assay kit for serum hydroperoxide, “Determiner LPO” (Kyowa Medexs) as in the previous report (Arakawa, 1995). This method was shown to be specific to lipid-hydroperoxide (Ohishi et al., 1985). Hydrogen peroxide and aldehydes such as malondialdehyde and formaldehyde are not detected by this method. The LDL solution from Sigma was determined to contain about 1.2 mm hydroperoxide. Thus, the incubation mixtures contained 36 µM hydroperoxide at the onset of an incubation.

**Effects of lipoprotein lipase and sodium deoxycholate on the sensitivity of LDL-hydroperoxide to sodium thiosulfate.** LDL-hydroperoxide was incubated with sodium thiosulfate in the presence of lipoprotein lipase and sodium deoxycholate. Fifteen microliters of LDL solution was mixed with 485 µl of 0.1 m PBS (pH = 7.0) containing 1 mm EDTA and 20 mm sodium thiosulfate. With or without sodium deoxycholate (1 mm) and lipoprotein lipase (EC 3.1.1.34, 5 U, from *alcaligenes* sp., Seikagakukogyo, Kokusho et al., 1982), the mixture was incubated at 25°C under darkness and the hydroperoxide content was monitored as above.

**Effects of sodium thiosulfate on cumene hydroperoxide.** Cumene hydroperoxide, a model organic hydroperoxide, was dissolved at 25 µM in 0.1 m PBS (pH = 7.0) containing 1 mm EDTA and 20 mm sodium thiosulfate. It was incubated at 25°C under darkness and the hydroperoxide content was monitored as above.
RESULTS AND DISCUSSION

The inhibitory profile of *P. separata* plasma melanization by sodium thiosulfate is shown in Fig. 1. Sodium thiosulfate over 20 mM (1–3 in Fig. 1) suppressed the melanization in 30 min incubation. A sodium thiosulfate concentration of higher than 50 mM (1, 2) was required to prevent the reaction over 6 h incubation. PTU (0.1%) was effective for 6 h (6). Since a white precipitate was formed in 6 h incubation at 100 mM sodium thiosulfate (1), the chemical was used below 50 mM in the following experiments. In the case of DDC, even a low dose (0.05 mM) was able to inhibit a blackening of the double-diluted plasma for 30 min, but not for 6 h. DDC higher than 0.1 mM was required to prevent the melanization for 6 h.

LDL-hydroperoxide (36 μM) was completely consumed in 2 h incubation with 1 mM DDC (closed circle in Fig. 2). Even lower doses of DDC at 0.1 mM (plus) and 0.05 mM

![Graph](image1)

**Fig. 1.** Inhibition of melanization by sodium thiosulfate and PTU. Diluted *P. separata* haemolymph plasma was incubated with 100 (1), 50 (2), 20 (3), 10 (4) or 0 (5) mM sodium thiosulfate, or with 0.1% PTU (6) at 25°C under darkness. Absorbance at 490 nm was read at 30 min (A) or 6 h (B). Distilled water served as a blank. The extent of melanization was also assessed as follows by eye: −, a pale yellowish solution; ±, a slightly brownish solution; +, a brownish solution without visible precipitate; +, a brownish solution with visible black precipitate; ±, a thoroughly blackened solution.

![Graph](image2)

**Fig. 2.** Effects of sodium thiosulfate and DDC on LDL-hydroperoxide. LDL-hydroperoxide (36 μM) was incubated with 0 (open circle), 5 (triangle), 20 (square), 50 (cross) mM sodium thiosulfate, or with 0.05 (reversed triangle), 0.1 (plus), 1 mM DDC (closed circle) at 25°C under darkness. Hydroperoxide content in the incubation mixture was monitored by the MB-HG method.
Fig. 3. Effects of lipoprotein lipase and sodium deoxycholate on the sensitivity of LDL-hydroperoxide against sodium thiosulfate. LDL-hydroperoxide (32 μM) was incubated with 1 mM sodium deoxycholate (triangle), 5 U lipoprotein lipase (cross), 1 mM sodium deoxycholate and 5 U lipoprotein lipase (closed circle) or none (open circle) in the presence of 20 mM sodium thiosulfate at 25°C under darkness. Hydroperoxide content in the incubation mixture was monitored by the MB-HG method.

Fig. 4. Degradation of cumene hydroperoxide by sodium thiosulfate. Cumene hydroperoxide (25 μM) was incubated with (cross) or without (circle) 20 mM sodium thiosulfate at 25°C under darkness. Hydroperoxide content in the incubation mixture was monitored by the MB-HG method.

(reversed triangle) caused a rapid decrease of the hydroperoxide. On the other hand, hydroperoxide decreased moderately in incubation with 20 (square) or 50 (cross) mM sodium thiosulfate. Five millimolar sodium thiosulfate (triangle) had little effect. Sodium thiosulfate is more hydrophilic than DDC. While DDC is soluble both in water and ethanol, sodium thiosulfate does not dissolve in ethanol. Thus, DDC could permeate a hydrophobic environment easily as compared with sodium thiosulfate. LDL is a globular molecule with a hydrophilic surface and a hydrophobic core (Chapman, 1980). A hydroperoxide group would be in the hydrophobic core, not exposed on the surface. Sodium thiosulfate would be less accessible to the hydroperoxide group in LDL. If an inaccessibility of the chemical has restricted the degradation of LDL-hydroperoxide, a digestion of LDL and an exposure of its internal hydroperoxide group should promote the decomposition of the hydroperoxide by sodium thiosulfate. Lipoprotein lipase is a digestive enzyme of plasma lipoproteins (KOKUSHO et al., 1982). In the presence of lipoprotein lipase, LDL-hydroperoxide decreased in an incubation with 20 mM sodium thiosulfate (cross in Fig. 3) faster than that without the
enzyme (open circle). Sodium deoxycholate, an enhancer of the enzyme, promoted the decrease (closed circle). Sodium deoxycholate had no effect when administered solely (triangle). These results are consistent with the idea that the inaccessibility of sodium thiosulfate to LDL-hydroperoxide has hindered the hydroperoxide degradation. Cumene hydroperoxide, a water-soluble organic hydroperoxide, was incubated with sodium thiosulfate. In this system, sodium thiosulfate was expected to access the hydroperoxide group freely. Hydroperoxide content in a mixture of cumene hydroperoxide with 20 mM sodium thiosulfate decreased in incubation (cross in Fig. 4). The decreasing profile resembled that of LDL-hydroperoxide incubated with sodium thiosulfate accompanied by lipoprotein lipase and sodium deoxycholate (closed circle in Fig. 3). The data suggest the potential of sodium thiosulfate to decompose the hydroperoxide under a freely accessible condition.

All these results suggest a possible use of sodium thiosulfate as a melanization inhibitor in experiments to explore the hydroperoxide species in insect haemolymph. Lipophorin is a major lipid transport molecule in insects, which exists in the haemolymph plasma (Chino, 1985). Lipophorin and vertebrate plasma lipoprotein resemble each other in their molecular configuration. Oxidative stresses occurring in the haemolymph should cause oxidative modifications of haemolymph components such as lipophorin. Twenty millimolar sodium thiosulfate could inhibit the melanization of diluted P. separata haemolymph for at least 30 min at 25°C. It would have little effect on the hydroperoxide hidden in a hydrophobic micro-environment, such as inside the plasma lipoprotein. DDC is unsuitable for such a purpose (Fig. 2). Experimental conditions should be determined in consideration of the phenoloxidase activity in the sample, temperature, duration of handling, and hydrophobicity of the hydroperoxide species and its distribution.

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REFERENCES


