Free Radical Research, Vol. 35, pp. 491–497
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Effect of Selenolipoic Acid on Peroxynitrite-dependent Inactivation of NADPH-cytochrome P450 Reductase

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Accepted by Professor H. Sies

(Received 7 November 2000; In revised form 14 March 2001)

Seleno-organic compounds are known as efficient "scavengers" of peroxynitrite (PN). Here we studied the protective effect of selenolipoic acid (SeLA), the seleno-containing analogue of lipoic acid, on peroxynitrite-dependent inactivation of NADPH-cytochrome P450 reductase. 3-Morpholinosydnonimine hydrochloride (SIN-1) was used as a source of peroxynitrite. The reductase was irreversibly inactivated by PN generated from SIN-1. The inactivation occurred with the rate constant of about $3\times10^4\,M^{-1}\,s^{-1}$. The presence of SeLA at low concentration (0.5 μM) led to synergistic increase of the reductase inactivation by PN. Our results suggest the formation of a reactive derivative of SeLA in the reaction of SeLA with PN, probably selenolseleninate, that mediates the aggravation of reductase inactivation. In the presence of SeLA, the inactivation was reversible under the action of thiols, allowing us to conclude that the observed action of SeLA may be considered as protective.

Keywords: Peroxynitrite; Selenolipoic acid; NADPH-cytochrome P450 reductase; Protection

INTRODUCTION

Peroxynitrite, $ONOO^-$, is a reactive species that can be generated in biological systems and organisms in several ways. It is produced by endothelial cells,[1] Kupffer cells,[2] neutrophils[3] and macrophages.[4] PN is known as a mediator of toxicity in inflammatory processes with strong oxidizing properties toward biological molecules, including sulfhydryls, lipids, amino acids and nucleotides. Tyrosine nitration of proteins by PN has also been demonstrated.[5,6] A number of enzymes, especially containing sulfhydryl groups, have been shown to be sensitive to oxidation by PN.[7,8] Biological systems have certain cellular mechanisms of protection against peroxynitrite generation.[9] However, under conditions of hyperproduction of PN additional protection against peroxynitrite might be required.

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Recently the ability of several seleno- and sulfur-organic compounds to protect against oxidation caused by peroxynitrite have been studied.[10,11] It has been demonstrated that seleno-containing compounds react with peroxynitrite more efficiently than their corresponding sulfur analogues.[10] Selenolipoic acid gained our attention as a seleno-containing analogue of α-lipoic acid, a well-known antioxidant able to scavenge reactive oxygen species as well as peroxynitrite.[11] Taking into account the reactivity of seleno-containing compounds with respect to PN it was of considerable interest to study the ability of selenolipoic acid to protect sulfhydryl groups of enzymes against oxidation by peroxynitrite.

We used NADPH-cytochrome P450 reductase to study the effect of selenolipoic acid on peroxynitrite-dependent inactivation of the enzyme. NADPH-cytochrome P450 reductase is the key enzyme of monooxygenase system responsible for the metabolism of a variety of xenobiotics. The reductase catalyses electron transfer from NADPH to cytochrome P450. It contains seven sulfhydryl groups, one or two of which are located in the NADPH-binding center.[12] Oxidation of these groups results in the loss of enzyme activity, hence it was reasonable to propose that the reductase is sensitive to PN. Previously[13,14] we have shown that the reductase is reversibly inactivated by millimolar concentrations of α-lipoic and selenolipoic acids via the modification of SH-groups at the active site. In the present study, we used micromolar concentrations of selenolipoic and α-lipoic acids to test their abilities to protect the NADPH-cytochrome P450 reductase against oxidation by peroxynitrite.

MATERIALS AND METHODS

Materials

Horse heart cytochrome c, diethylenetriaminepentaacetic acid (DTPA), dihydrorhodamine 123 (DHR), glutathione (GSH) and superoxide dismutase (SOD) were purchased from Sigma (USA). Selenolipoic and α-lipoic (LA) acids were from ASTA Medica (Germany). Dithiothreitol (DTT) was from Fluka (Switzerland). NADPH and $NADP^+$ were from Reanal (Hungary). 3-Morpholinosydnonimine hydrochloride (SIN-1) was purchased from Molecular Probes, Inc., Eugene OR. NADPH-cytochrome P450 reductase (FP) isolated from rat hepatic microsomes was kindly provided by Dr A. Grishanova (Institute of Molecular Biology and Biophysics, Novosibirsk). Other chemicals were of the highest grade commercially available.

Assay of NADPH-cytochrome P450 Activity

The activity of NADPH-cytochrome P450 reductase was determined spectrophotometrically[15] from the reduction rates of cytochrome c at 550 nm in the presence of 1 mM NADPH in 0.3 M phosphate-buffered saline (PBS), pH 7.4. The spectrophotometrical measurements were performed on an Ultraspec (LKB, Sweden).

Peroxynitrite Generation by SIN-1: Inhibition of DHR Oxidation by SeLA and α-LA

We used SIN-1 as a source of peroxynitrite. Peroxynitrite formation from SIN-1 results from the simultaneous production of superoxide and nitric oxide, which in turn, react at a near diffusion-limited rate.[16] Peroxynitrite formation from SIN-1 was assayed spectrophotometrically by continuous monitoring of rhodamine formation from DHR at 500 nm at room temperature (as described in Ref. [7]). The reaction solution consisted of 50 μM DHR, 0.1 mM DTPA, and 1 mM SIN-1 in 0.3 M PBS, pH 7.4.

In order to test the reactivity of SeLA and α-LA towards PN, the rate of rhodamine formation was measured in the absence and in the presence of these compounds. α-LA (up to 9 mM) or SeLA (up to 0.3 mM) were added to the reaction solution described above.

Inactivation of NADPH-cytochrome P450 Reductase by SIN-1

We have observed that the rate of PN production from SIN-1 becomes constant after an initial period as was reported previously.[17] Therefore the stock solution of SIN-1 (10 mM) in 0.3 M PBS, pH 7.4, with 0.1 mM DTPA was allowed to stay at room temperature until maximum rate of PN generation was reached. For the enzyme inactivation stock solution of SIN-1 was added to FP (0.3 μM) to the final concentrations of 1 mM or 0.1 mM (see figure captions). Aliquots were removed from reaction mixtures at different times, diluted 40-fold and assayed for reductase activity as described above. In the control experiment, it was shown that the stable decomposition products of SIN-1 had no effect on the reductase activity.

Optical Absorption Spectra

Spectra were recorded with a 1-cm path length cell with DV-GD (Beckman) spectrometer.

RESULTS

Inactivation of NADPH-cytochrome P450 Reductase by SIN-1

Prior to the study of protective ability of SeLA we have verified that the reductase is sensitive to oxidation under the action of PN. SIN-1 was used for continuous generation of peroxynitrite in our experiments. Peroxynitrite production was determined by its ability to oxidize dihydrorhodamine. The maximum rate of peroxynitrite formation determined from the linear part of the kinetics of dihydrorhodamine oxidation was found to be 0.35 μM/min. A continuous exposure of NADPH-cytochrome P450 reductase to SIN-1 led to significant inactivation of the enzyme (Fig. 1). In the control experiment FP was incubated under identical conditions in the presence of SOD, which prevents the formation of PN from SIN-1.[17] Addition of SOD to the incubation mixture completely protected the reductase from inactivation by SIN-1 (Fig. 1), confirming that it was peroxynitrite that affected the reductase activity in our experimental conditions. The rate constant of reductase inactivation by PN was estimated by assuming quasi steady state approximation for PN concentration. Under this approximation, the steady state concentration of PN was estimated based on the observed rate of PN generation from SIN-1 (0.35 μM/min), the known rate of spontaneous PN decomposition in phosphate buffer ($0.35\,s^{-1}$)[8] and was found to be equal to 20 nM. Based on this value the pseudo-first-order rate constant for the reductase inactivation by PN was estimated to be equal to $3\times10^{4}\,M^{-1}s^{-1}$.

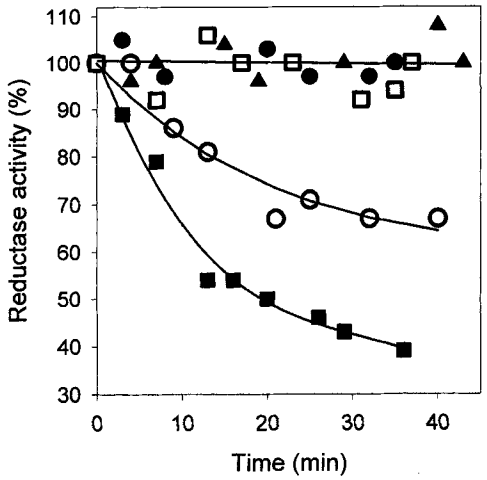

FIGURE 1 Solid symbols: inactivation of FP by SIN-1. FP (0.3 μM) was incubated with 1 mM SIN-1, either alone (■) or together with 720 units/ml of SOD (●) at room temperature in 0.3 M PBS, pH 7.4 containing 0.1 mM DTPA. Aliquots were removed at indicated times, diluted 40-fold and assayed for FP activity as described in "Materials and methods". In the control experiment FP was incubated under identical conditions without SIN-1 (▲). This incubation did not result in loss of FP activity. Open symbols: protective effect of $NADP^+$ on FP inactivation by SIN-1. SIN-1 (0.1 mM) was added to FP (0.3 μM) in the presence (□) or absence (○) of 1 mM $NADP^+$ in 0.3 M PBS, pH 7.4, containing 0.1 mM DTPA at room temperature. Aliquots were removed at indicated times, diluted 40-fold and assayed for FP activity as described in "Materials and Methods".

Protective Effect of $NADP^+$ On the Reductase Inactivation by SIN-1

In order to elucidate the process of FP inactivation by PN, a competitive inhibitor of the reductase,

$NADP^+$, was used. As shown in Fig. 1, addition of $NADP^+$ to the reaction mixture prior to addition of SIN-1, protected the reductase against the effect of PN. The result indicates that reductase inactivation under the action of PN occurred due to the oxidation of amino acid residues at the active site, most likely essential sulfhydryl groups of cysteines. In the case of oxidation of these SH-groups of the reductase to disulfide bond the enzyme activity can be restored by addition of DTT according to the reaction of thiol–disulfide exchange.[13] However, addition of DTT to the reductase after its incubation with SIN-1 did not restore the enzyme activity (Fig. 2). The irreversibility of reductase inactivation by PN suggests the higher oxidation of sulfhydryl groups rather than formation of disulfides.[18]

Effect of Selenolipoic Acid on the Reductase Inactivation by SIN-1

In order to study the protective effect of SeLA on PN-induced reductase inactivation, we used a low concentration of SeLA (0.5 μM), which itself did not affect the enzyme activity (Fig. 3). We suggested that in the system of the reductase and SeLA PN might react with SeLA rather than with SH-groups of the reductase, thus preventing reductase inactivation. However, incubation of the reductase with the mixture of SIN-1 and SeLA resulted in unexpected aggravation of enzyme inactivation compared to the case of SIN-1 alone (Fig. 3). Addition of SOD to the reductase before exposure to the mixture of SeLA and SIN-1 completely prevented enzyme inactivation (Fig. 3). These results suggest the formation of a highly reactive intermediate in the reaction of SeLA with PN, which is able to inactivate the reductase efficiently. In order to verify the formation of intermediate, we incubated SeLA with SIN-1 for up to 35 min. Then SOD was added to that mixture to stop the reaction of PN with SeLA. As shown in Fig. 4, the resultant mixture caused more extensive inactivation of the reductase in comparison with either

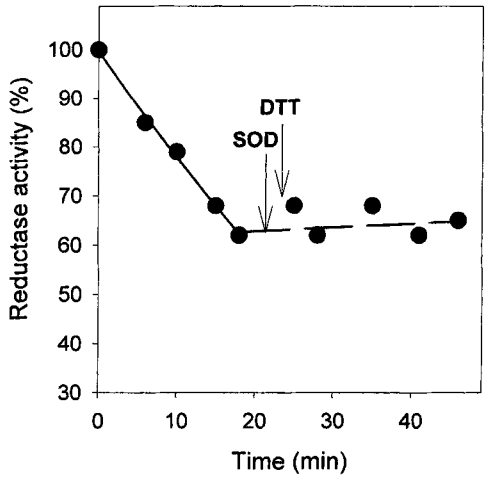

FIGURE 2 Effect of DTT on FP activity after inactivation by 1 mM SIN-1. FP (0.3 μM) was incubated with 1 mM SIN-1 in 0.3 M PBS, pH 7.4 with 0.1 mM DTPA at room temperature. To stop the formation of peroxynitrite, SOD (720 U/ml) was added to the reaction mixture at indicated times prior to the addition of DTT. Then DTT was added to the incubation mixture up to 1.25 mM. Aliquots were removed at intervals shown, diluted 40-fold and assayed for FP activity as described in "Materials and Methods". In the control experiment 0.03 mM DTT did not reduce cytochrome c.

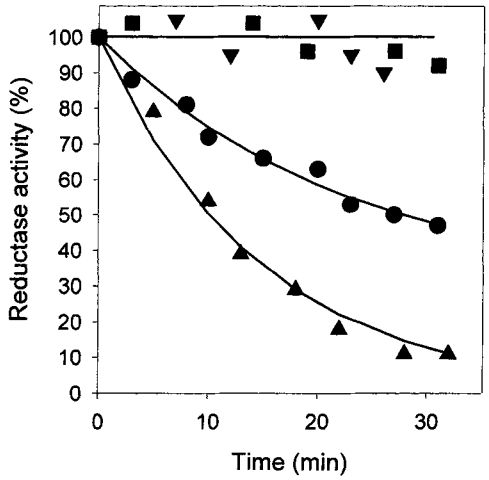

FIGURE 3 Cooperative effect of SeLA and SIN-1 on FP activity. Protective effect of SOD. FP (0.3 μM) was incubated with 1 mM SIN-1 in the presence (▲) or in the absence (●) of 0.5 μM SeLA in 0.3 M PBS, pH 7.4 with 0.1 mM DTPA at room temperature. In the control experiment FP was incubated with 0.5 μM SeLA alone (■). Under identical conditions SeLA (0.5 μM) and 1 mM SIN-1 were added to the FP (0.3 M) in the presence of SOD, 720 U/ml (▼). Aliquots were removed at intervals shown, diluted 40-fold and assayed for FP activity as described in "Materials and Methods".

SeLA or SIN-1 alone. In the case of the reductase inactivation by the mixture SIN-1+SeLA the activity of the reductase was significantly recovered by DTT (Fig. 4) as well as by GSH (data not shown), indicating the formation of a disulfide-like bond at the active site of the enzyme addition. The absorption spectra of the reaction mixture, containing SIN-1 and SeLA, were obtained (Fig. 5). Fig. 5 shows the appearance and increase of the peak in the vicinity of 300 nm while the peak corresponding to SeLA (λ=420 nm) decreases. If SOD (720 U/ml) was present in the reaction mixture the absorption band at 300 nm was not observed (data not shown). These observations confirm the formation of the intermediate in the reaction of SeLA with PN.

In similar experiments SeLA was replaced by the same concentration (0.5 μM) of α-LA. In this case, we did not observe neither protective nor aggravating effects on reductase inactivation.

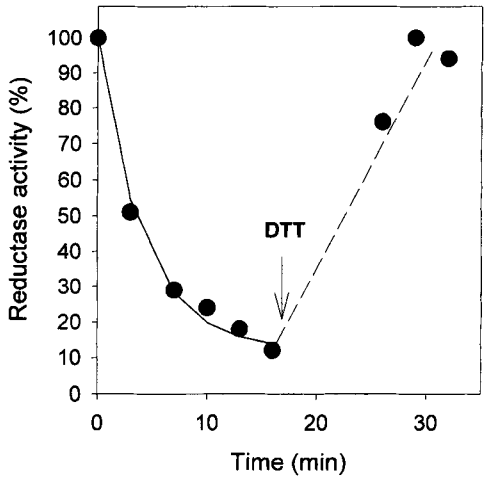

FIGURE 4 Recovery of FP activity under the action of DTT after inhibition by SeLA + SIN-1. SeLA (0.5 μM) was pre-incubated with SIN-1 (1 mM) in 0.3 M PBS, pH 7.4 with 0.1 mM DTPA at room temperature during 35 min. The reaction was stopped by addition of SOD (720 U/ml), then FP (0.3 μM) was added to the reaction mixture. After 17 min of incubation DTT was added to the solution up to 1.25 mM. Aliquots were removed at intervals shown, diluted 40-fold and assayed for FP activity as described in "Materials and Methods". In control experiment 0.03 mM DTT did not reduce cytochrome c.

These results are in agreement with the data obtained from DHR oxidation by PN generated by SIN-1 in the presence of SeLA or α-LA SeLA (0.3 mM) decreased oxidation of DHR by 68% while higher concentrations of α-LA (up to 9 mM) did not show any significant effect (data not shown). The same reduction of DHR oxidation by α-LA was observed only at the concentration of 10^{-1} M that is three order of magnitude higher than the concentration of SeLA. It should be noted that the reversibility of FP inactivation by PN in the presence of α-LA can be reached but only at its significantly higher concentration (10 μM) than SeLA (0.5 μM).

DISCUSSION

Recent studies have demonstrated that seleno-organic compounds are efficient "scavengers" of PN and can carry out a biological function of defence against peroxynitrite.[9] It was of special interest to test the protective ability of selenolipoic acid and to compare it with its sulfur-containing analogue, the well-known antioxidant α-lipoic acid.

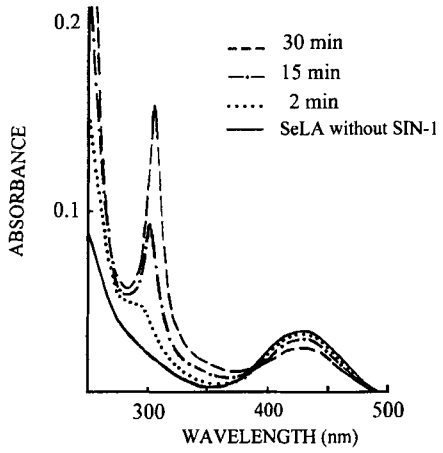

FIGURE 5 Oxidation of SeLA by PN generated by SIN-1. Optical absorption spectra of the reaction mixture containing 0.5 mM SIN-1 and 0.5 mM SeLA in 0.3 M PBS, pH 7.4 with 0.1 mM DTPA were taken after 2, 15 and 30 min of incubation. Spectra were recorded with reference to 0.5 mM SIN-1 in 0.3 M PBS, pH 7.4 with 0.1 mM DTPA.

As expected, PN generated by SIN-1 effectively inactivates the reductase (Fig. 1), mostly due to oxidation of sulfhydryl groups at the enzyme active site. The inactivation was not reversible by DTT (Fig. 2), suggesting higher oxidation of sulfhydryl groups, different from formation of disulfides, e.g. sulfinic or sulfonic acid.[18] Reductase inactivation by PN occurred with the rate constant of about $3 \times 10^4 M^{-1} s^{-1}$, which is similar to the rates at which PN inactivates sulfhydryl groups of albumin[18] and alcohol dehydrogenase.[7] On the basis of the known higher reactivity of seleno-organic compounds towards PN in comparison with sulfur analogues, we suggested that SeLA could protect the reductase against PN more efficiently than α-LA. In support of this idea, we have shown that low concentrations of SeLA effectively protected DHR against oxidation by PN, while α-LA did not. However, in the experiments with the reductase, we did not observe the protective effect of SeLA (Fig. 3). Moreover, the presence of SeLA led to a synergistic increase of reductase inactivation under the action of PN (Fig. 3). The same concentration of α-LA neither prevented nor aggravated PN-dependent inactivation of the reductase. The effect of aggravation of PN-dependent enzyme inactivation has been reported previously by Whiteman *et al.*[8] for several thiols at low concentrations. The authors proposed that this aggravation was mediated by sulfur radicals, formed in the reaction of certain thiols/disulfides with PN.[8] Based on our data we suggest that synergistic increase of the reductase inactivation is also mediated by a reactive intermediate, formed in the reaction of SeLA and PN. In support of this suggestion, we have shown that pre-incubation of SeLA with SIN-1 up to 35 min resulted in the formation of the product, which effectively inactivated the reductase. It is of importance that in the presence of SeLA the reductase inactivation by PN was reversible (Fig. 4), whereas the reductase inactivation without addition of SeLA was irreversible (Fig. 2). The reversibility of enzyme inactivation by low weight thiols (DTT, GSH) indicates the formation of a disulfide-like bond at the enzyme active site with a highly reactive derivative of SeLA. In order to elucidate this kind of derivative, the optical absorption spectra of the reaction mixture, containing SeLA and SIN-1, were investigated. The spectra confirmed the formation of the derivative of SeLA, which is seen from the appearance of a new absorption band with maximum at 300 nm and the presence of an isobestic point (Fig. 5). In the presence of SOD the peak at 300 nm did not appear. Earlier, it has been shown by Bergson[19] that diselenides oxidation leads to the formation of the intermediate, selenolseleninate, with an absorption band in the vicinity of 300 nm.[19] According to its chemical structure, selenolseleninate (Fig. 6) is able to form a disulfide-like bond with thiol groups of proteins. This fact and the data obtained allow us to propose that selenolseleninate is formed in the reaction of SeLA and PN (Fig. 6), although more work is needed to establish this.

Thus, SeLA at low concentration, does not completely protect the reductase and even

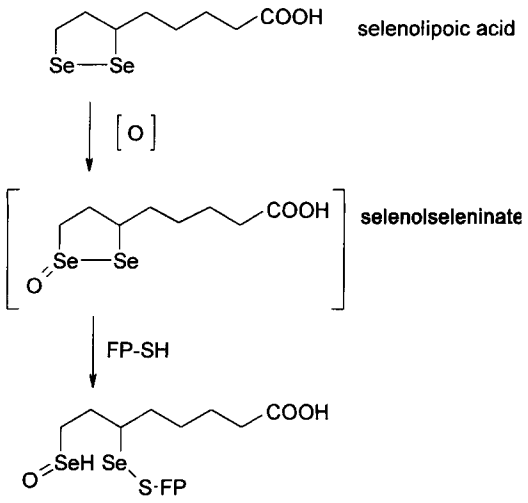

FIGURE 6 Hypothetical scheme of the chemical modification of FP under the action of SeLA + SIN-1. FP-SH indicates sulfhydryl group at active site of the reductase. Selenolseleninate is suggested to be formed in the reaction of SeLA with PN.

synergistically increases PN-dependent inactivation of the enzyme. However, in the presence of SeLA, the inactivation becomes reversible upon addition of DTT or GSH. In the terms of reversibility of PN-dependent oxidation of the reductase the observed mechanism of action of SeLA against PN may be considered as protective. In contrast to that data, low concentration of α-LA did not protect the reductase from inactivation by PN. Since the monooxygenase system mediates the metabolic formation of NO from organic nitrates, with simultaneous production of the superoxide radical,[20,21] the formation of PN in the monooxygenase system may be suggested. Therefore, the obtained data on the inactivation of NADPH-cytochrome P450 reductase may represent a possible mechanism of PN-mediated toxicity in the monooxygenase system.

Acknowledgements

We thank Dr A. Grishanova for the NADPH-cytochrome P450 reductase. S.S. was supported by a grant from the international Soros Science Education Program and Russian Foundation of Basic Researches, grant 99-04-49921.

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