Action of nitric oxide as an antioxidant against oxidation of soybean phosphatidylcholine liposomal membranes

Katsunobu Hayashi, Noriko Noguchi, Etsuo Niki*

Research Center for Advanced Science and Technology (RCAST), University of Tokyo, 4-6-1 Komaba, Meguro, Tokyo 153, Japan

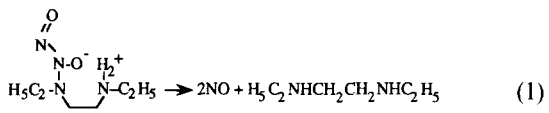
Received 25 May 1995; revised version received 16 June 1995

Abstract To elucidate the protective role of nitric oxide (NO) against lipid peroxidation, the effect of NO donor on the formation of lipid hydroperoxide and consumption of α-tocopherol in the oxidation of soybean phosphatidylcholine liposomal membranes was studied. The oxidation was induced by either aqueous or lipophilic peroxyl radicals generated by the hydrophilic or lipophilic azo compound, respectively. It was found that NO acted as a potent antioxidant by scavenging peroxyl radicals rapidly. It was also found that NO was capable of penetrating multilamellar membranes to scavenge lipid peroxyl radicals and spare α-tocopherol.

Key words: Antioxidant; Free radical; Lipid peroxidation; Nitric oxide

1. Introduction

Nitric oxide (NO) has received much attention because of its diverse biological activities [1]. Its structure is simple, but its action and function are complex. It is an endothelial-derived relaxation factor which modulates blood pressure and it also mediates a variety of biological actions ranging from vasodilation, neurotransmission, inhibition of platelet adherence and aggregation, and killing of pathogens [1,2]. It is known that NO plays both salutary and toxic role. For example, it has been reported that NO mediates tissue injury but also prevents damage during the ischemia-reperfusion event [3]. It has been also found that NO together with superoxide induces lipid peroxidation [4] and oxidative modification of low-density lipoprotein (LDL) [5–9], but that it also inhibits lipid peroxidation [6,10–12], the total effect of NO being critically dependent on relative concentrations of individual reactive species [11]. The rate constants for the reaction of NO with the peroxyl radicals derived from alcohols were obtained as 10^9 $M^{-1}s^{-1}$ [13]. The basic chemistry of NO is not well understood yet and the present study was undertaken to elucidate the action of NO as a radical-scavenging antioxidant and compare it with those of ascorbic acid (vitamin C) and α-tocopherol (vitamin E), major hydrophilic and lipophilic antioxidants in vivo. The oxidation of soybean phosphatidylcholine (PC) liposomes induced by either hydrophilic or lipophilic azo compound was chosen as the oxidation system considering following advantages [14]: (1) the peroxyl radicals are responsible for both chain initiation and propagation, which makes it possible to measure the antioxidant action of NO specifically as a scavenger of peroxyl radical; (2) the azo compounds enable us to generate initiating radicals at a constant rate at a controlled site; and (3) soybean PC contains high concentrations of linoleic acid moieties which makes it easy to follow the rate of oxidation quantitatively. NO was generated from NO-releasing compound, 1-hydroxyl-2-oxo-3-(*N*-ethyl-2-aminoethyl)-3-ethyl-1-triazene (NOC12), which releases NO spontaneously at ambient temperatures without the requirement of enzyme activation or biotransformation (Eq. 1) [15].



$$H_5C_2\text{-}N(\text{-}N(O^-)\text{-}H_2^+)\text{-}N\text{-}C_2H_5 \rightarrow 2NO + H_5C_2NHCH_2CH_2NHC_2H_5 \quad (1)$$

2. Materials and methods

2.1. Materials

NOC12 was supplied from Dojindo Laboratories and used as received. Soybean PC was purchased from Sigma (St Louis, MO) and purified before use by column chromatography [16]. 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) and 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) were obtained from Wako Pure Chemical Ind. (Osaka, Japan). (2*R*,4′*R*,8′*R*)-α-Tocopherol kindly supplied from Eisai Co. (Tokyo, Japan) and commercial ascorbic acid were used without further purification.

2.2. Oxidation procedure

Soybean PC liposomal membranes were prepared as reported previously [16]. Briefly, soybean PC and lipophilic additives, such as AMVN and α-tocopherol, when used were dissolved into methanol in a pear-shaped flask and the solvent was evaporated slowly to obtain a thin film on the flask wall. A phosphate-buffered saline (PBS, pH 7.4) containing 100 μM ethylenediaminetetraacetic acid (EDTA) was added and the film was peeled off to obtain a white milky suspensions of multilamellar vesicles. Water-soluble additives, such as AAPH, NOC12 and ascorbic acid, were added when required as a PBS solution containing 100 μM EDTA after preparation of the liposomes. The oxidation was performed at 37°C in air. An aliquot of the reaction mixture was taken out periodically and analysed as described below [17].

2.3. Analysis of products and antioxidant

The oxidation of soybean PC liposomes gives conjugated diene hydroperoxides almost quantitatively [16]. The formation of PC hydroperoxides was followed with an HPLC by measuring conjugated diene at 234 nm absorption [11]. The silica gel column, LC-Si (25 cm) (Supelco, Tokyo, Japan) was used and methanol/40 mM phosphate buffer (90:10 v/v) was used as an eluent at a rate of 1.0 ml/min. The consumption of α-tocopherol was followed with an HPLC equipped with an electrochemical detector using LC-18 column (Supelco) and methanol/*tert*-butyl alcohol (90:10 v/v) containing 50 mM $NaClO_4$ as an eluent at a rate of 1.0 ml/min. The electrochemical detector (Kotaki ECP-1, Chiba, Japan) was set at +800 mV.

*Corresponding author. Fax: (81) (3) 3481 4574.

Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); EDTA, ethylenediamine-tetraacetic acid; LDL, low-density lipoprotein; NOC12, 1-hydroxyl-2-oxo-3-(*N*-ethyl-2-aminoethyl)-3-ethyl-1-triazene; PBS, phosphate-buffered saline; PC, phosphatidylcholine.

SSDI 0014-5793(95)00786-5

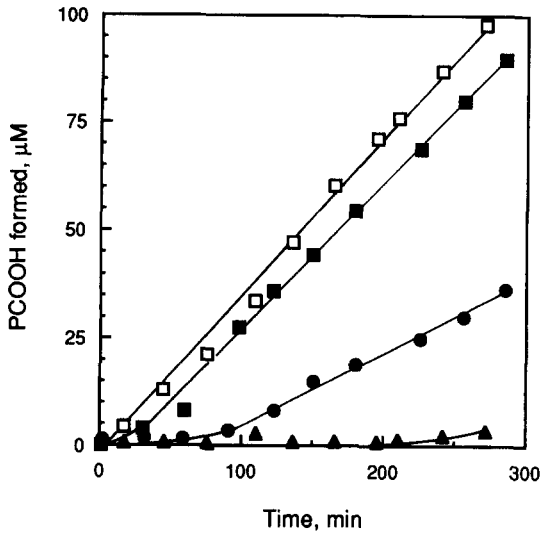


Fig. 1. Effect of NOC12 on the oxidation of soybean PC liposomes induced by AAPH. The oxidation of soybean PC (5.15 mM) multilamellar vesicles was carried out at 37°C in air in the presence of AAPH (0.50 mM) with and without NO donor NOC12 and the accumulation of PC hydroperoxides (PCOOH) was followed with HPLC as described in Section 2. The initial concentration of NOC12 was □: 0; ■: 2; ●:5; ▲: 20 μM.

3. Results

The oxidation of soybean PC liposomes induced by either AAPH or AMVN gives PC hydroperoxides quantitatively at a constant rate. Fig. 1 shows that the rate of accumulation of PC hydroperoxides was 6.35×10^{-9} M/s. The rate of aqueous radical flux from 0.50 mM AAPH in the aqueous phase is calculated as 0.65×10^{-9} M/s [14]. Thus, the kinetic chain length is obtained as 9.8. As shown in Fig. 1, NOC12 suppressed the oxidation initially and gave a clear lag time, which increased with increasing NOC12 concentration.

Fig. 2 shows the results of oxidation of soybean PC liposomes (multilamellar vesicles) induced by lipophilic AMVN in the absence and presence of NOC12. AMVN was incorporated into the membranes by mixing with soybean PC before preparation of the film and hence AMVN must be present in every membrane. In the absence of any antioxidant, PC hydroperoxides were accumulated at a constant rate without any lag phase (□ in Fig. 2). Ascorbic acid (20 μM) added to the reaction mixture at 220 min incubation did not suppress the oxidation appreciably (⊞ in Fig. 2). When 5 μM NOC12 was added, the oxidation was suppressed intially and then proceeded at the similar rate as that without NOC12 after a lag period of about 60 min (■ in Fig. 2). When 20 μM NOC12 was then added into the reaction mixture after the lag time at 250 min incubation, the oxidation was suppressed almost completely again (◆ in Fig. 2). Little oxidation was observed in 180 min when 20 μM NOC12 was added initially.

The effect of NO on the oxidation of soybean PC liposomes inhibited by α-tocopherol was also studied (Fig. 3). α-Tocopherol was incorporated into the membranes simultaneously with AMVN. The oxidation was suppressed by α-tocopherol efficiently. α-Tocopherol was consumed at a constant rate and, when it was depleted, the lag phase was over and a fast oxidation took place. When 2 μM NOC12 was added, the lag phase was prolonged and the rate of consumption of α-tocopherol was decreased. A higher concentration of NOC12 (20 μM) gave more profound effect, i.e., little oxidation was observed in 200 min and α-tocopherol was spared more markedly. To test the role of decomposition product from NOC12 as an antioxidant, the solution containing NOC12 was incubated at 37°C for 3 days and then added to the reaction mixture. The oxidation was not suppressed appreciably by this solution (NOC12 equivalent to 50 μM), suggesting that *N,N'*-diethylethylenediamine does not act as an antioxidant.

4. Discussion

The above results clearly show that the NO donor NOC12 suppresses the oxidations of soybean PC liposomal membranes effectively. Upon thermal decomposition, NOC12 gives 2 molecules of NO and *N,N'*-diethylethylenediamine [15]. As described above, *N,N'*-diethylethylenediamine was virtually ineffective in suppressing the oxidation, and it may safely be assumed that NO was responsible for the inhibition of oxidation.

The inhibitory effect of NO on the oxidation of lipids has been reported by several groups. The effect of NO on the oxidation of LDL has been studied extensively. Dee et al. [18] found that NO was capable of either enhancing or suppressing the ferryl myoglobin-mediated oxidation of LDL depending on the relative concentrations of NO to hydrogen peroxide. Jessup et al. [6] have found that NO exerts a protective role in preventing the oxidative modification of LDL by macrophages, although it can oxidize LDL together with superoxide. Hogg et al. [10] also observed the inhibitory effect of NO on the oxidation of LDL and proposed that NO inhibited oxidation by scavenging carbon-centered and peroxyl radicals. Padmaja and Hire [13] have measured the absolute rate constant for the reactions of NO and peroxyl radicals derived from alcohols by laser-flash photolysis with kinetic spectrophotometry and found that the reactions are very fast with the rate constant of $> 10^9$ $M^{-1}s^{-1}$. More recently, Rubbo et al. [11] have studied the effect of NO on soybean PC liposomal membranes induced by superoxide, hydrogen peroxide and peroxynitrite and found that NO can act as a radical scavenging antioxidant. They in fact identified the products formed by the interactions of NO with lipid peroxyl and alkoxyl radicals [11].

The results of the present study are in agreement with the previous findings and clearly show that NO acts as a peroxyl radical scavenging antioxidants. The oxidation system employed in this study is simple and only peroxyl radicals are involved in both chain initiation and propagation. That is, the azo compound undergoes thermal decomposition to give carbon-centered radical, which reacts quite rapidly with oxygen to give peroxyl radical. This radical attacks bisallylic hydrogen of phosphatidylcholine and induces chain propagation, in which the lipid peroxyl radicals act as chain carrying species. Thus, the inhibition of oxidation of AAPH or AMVN-initiated oxidation of soybean PC liposomes by NO is ascribed exclusively to the scavenging of peroxyl radicals (derived from the azo compound and/or lipid) by NO.

This study gives us new interesting information. The results in Fig. 1 show that NO scavenges aqueous peroxyl radicals derived from AAPH and/or lipophilic peroxyl radicals derived from PC, but they do not prove if NO is really capable of scavenging lipophilic radicals. The oxidation of soybean PC

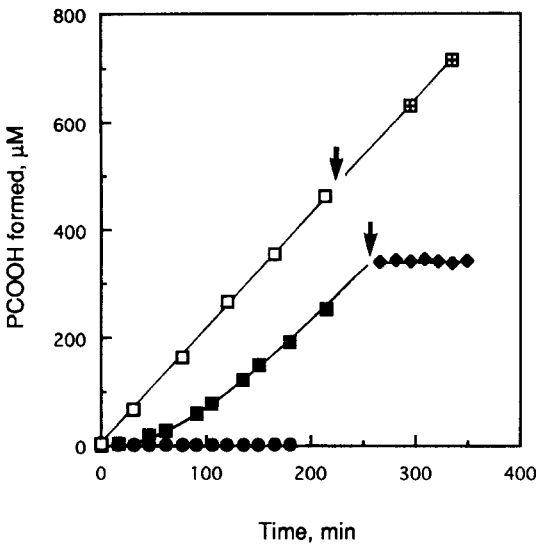


Fig. 2. Effect of NO donor NOC12 on the oxidation of soybean PC liposomes induced by AMVN. The oxidation of soybean PC (5.15 mM) multilamellar vesicles was induced by AMVN (1 mM) at 37°C in air in the absence and presence of NOC12 and the formation of PC hydroperoxides (PCOOH) was measured as described in Section 2. NOC12 was added as a PB solution after preparation of liposomes. The initial concentrations of NOC12 was □: 0; ■: 5 μM; ●: 20 μM. Ascorbate (20 μM) and NOC12 (20 μM) was added to the reaction mixture at the point indicated by an arrow and PCOOH was measured, the results being shown by ⊞ and ◆, respectively.

multilamellar liposomal membranes induced by AMVN incorporated into the membranes proceeds at every membrane to give PC hydroperoxides. Fig. 2 shows that NOC12 added into the aqueous phase after preparation of the membranes suppressed the oxidation almost completely, implying that NO is capable of penetrating the membranes and scavenging radicals in every membrane. On the other hand, ascorbic acid (a potent, hydrophilic radical scavenging antioxidant) added similarly did not suppress the oxidation, suggesting that ascorbic acid located outside multilamellar vesicles is not capable of scavenging lipophilic radicals within the membranes efficiently. Furthermore, the results in Fig. 3 show that NOC12 added into the aqueous phase outside multilamellar vesicles spared α-tocopherol incorporated into membranes, suggesting that NO released from NOC12 outside the membranes competes with α-tocopherol well and spares it.

α-Tocopherol scavenges peroxyl radical in solution with a rate constant of about 10^6 $M^{-1}s^{-1}$ [19,20]. The antioxidant activity of α-tocopherol is reduced in the membranes [21] and the rate constant for scavenging PC peroxyl radical in liposomal membranes is reported as 5.8×10^3 $M^{-1}s^{-1}$ [22]. If we assume the rate constants for scavenging peroxyl radicals by NO and α-tocopherol in the membranes as 10^9 [13] and 10^4 M^{-1} s^{-1} [22], respectively, then it follows that NO can compete equally well with α-tocopherol at much smaller concentration. If we further assume the concentration of α-tocopherol in plasma to be 20 μM, then NO of as low as 0.2 nM is estimated to be as effective as α-tocopherol. Ascorbate also scavenges aqueous peroxyl radical rapidly with a rate constant of about 10^5~10^6 M^{-1} s^{-1} [20,23]. If we assume the rate constant for scavenging peroxyl radical and concentration of ascorbate in the plasma as 5×10^5 M^{-1} s^{-1} and 50 μM, respectively, then it is calculated that 25 nM NO is as effective as ascorbate in scavenging aqueous radicals. From these calculations, one might speculate that NO may under some conditions predominate over α-tocopherol as an antioxidant. It may be worth noting that NO is lipophilic, the lipid:water partition coefficient being 8:1 [11]. However, it should be pointed out that, as discussed above, NO can move between the membranes very freely.

The antioxidant efficacy of NO depends not only on its rate constant for scavenging peroxyl radical but also on the stability of the reaction products and other competing reactions. The reaction of NO with peroxyl radical gives nitrosoperoxo compound or organic peroxynitrite [11,13], which may

$$LO_2^{\bullet} + {}^{\bullet}NO \rightarrow LOONO \rightarrow [LO^{\bullet} + {}^{\bullet}NO_2] \rightarrow \text{free } LO^{\bullet} + {}^{\bullet}NO_2 \quad (2)$$

$$\rightarrow LONO_2 \quad (3)$$

decompose to give alkoxyl and nitrogen dioxide radicals (Reaction 2) and/or stable organic nitrite (Reaction 3) [13,24,25]. If the nitrosoperoxo compound should give alkoxyl radical, this will end up in the chain branching and NO does not act as an antioxidant unless NO scavenges the alkoxyl radical efficiently. Such reactions have been studied extensively by Pryor et al. [24,25] and the formation of free alkoxyl radical was confirmed by spin trapping [24]. The stability of nitrosoperoxo compound depends on the solvent [13] but its stability and fate in the membranes and lipoproteins are not known. The homolysis of peroxynitrous acid to give free hydroxyl and nitrogen dioxide radicals has been shown to be unfavorable from thermodynamic and kinetic considerations [26,27]. The homolytic scission of organic peroxynitrite to give alkoxyl radical and nitrogen dioxide should be more feasible than that of peroxynitrous acid, but the present study suggests that organic peroxynitrite is stable under the conditions employed here.

It is known that NO reacts rapidly with superoxide at diffu-

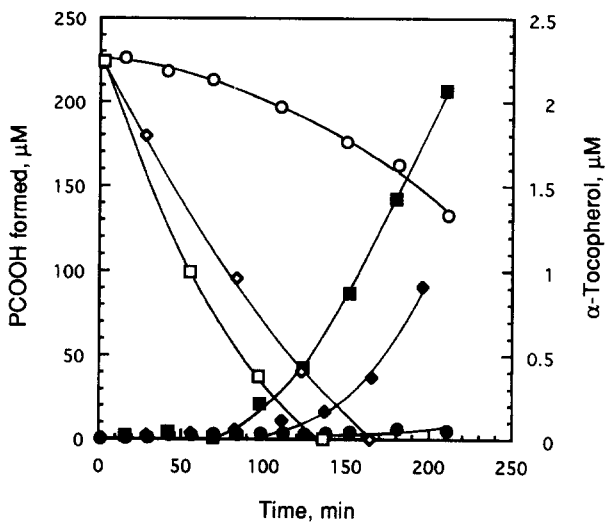


Fig. 3. Oxidation of soybean PC liposomes induced by AMVN in the presence of α-tocopherol and NO donor NOC12. AMVN (1.0 mM) and α-tocopherol (2.2 μM) was incorporated simultaneously into soybean PC multilamellar liposomal membranes (5.15 mM), which were subjected to oxidation by incubation at 37°C in air with and without NOC12. The formation of PC hydroperoxides (PCOOH) (solid symbols) and consumption of α-tocopherol (open symbols) was measured with an HPLC as described in Section 2. The initial concentration of NOC12 was ■, □: 0; ◆, ◇: 2 μM; ●, ○: 20 μM.

sion control rate to give peroxynitrite [28,29], which induces the oxidations of various biological molecules. Thus, the presence of superoxide will diminish the antioxidant nature of NO but instead enhances prooxidant function of NO.

In conclusion, the present study shows clearly that NO is capable of acting as a potent antioxidant against lipid peroxidation by scavenging peroxyl radical. NO is diffusible through membranes and it might even predominate over α-tocopherol under some circumstances but the total antioxidant efficacy of NO depends on its concentration, the relative importance of the competing reactions, such as with superoxide, and stability of the organic peroxynitrite.

References

[1] Moncada, S. and Higgs, E.A. (1991) Eur. J. Clin. Invest. 21, 361–374.
[2] Lipton, S.A., Choi, Y.-B., Pan, Z.-H., Lei, S.Z., Chen, H.-S.V., Sucher, N.J., Loscaizo, J., Singel, D.J. and Stamier, J.S. (1993) Nature 364, 626–632.
[3] Dawson, V.L., Dawson, T.M., London, E.D., Bredt, O.S. and Sayder, S.H. (1991) Proc. Natl. Acad. Sci. USA 88, 6368–6371.
[4] Radi, R., Beckman, J.S., Buch, K.M. and Freeman, B.A. (1991) Arch. Biochem. Biophys. 288, 481–487.
[5] Darley-Usmar, V.M., Hogg, N., O'Leary, V.J., Wilson, M.T. and Moncada, S. (1992) Free Rad. Res. Comm. 17, 9–20.
[6] Jessup, W., Mohr, D., Gieseg, S.P., Dean, R.T. and Stocker, R. (1992) Biochim. Biophys. Acta 1180, 73–82.
[7] Hogg, N., Darley-Usmar, V.M., Wilson, M.T. and Moncada, S. (1993) FEBS Lett. 326, 199–203.
[8] Graham. A., Hogg, N., Kalyanaraman, B., O'Leary, V., Darley-Usmar, V. and Moncada, S. (1993) FEBS Lett. 330, 181–185.
[9] Wang, J.-M., Chow, S.-N. and Lin, J.-K. (1994) FEBS Lett. 342, 171–175.
[10] Hogg, N., Kalyanaraman, B., Joseph, J., Struck, A. and Parthasarathy, S. (1993) FEBS Lett. 334, 170–174.
[11] Rubbo, H., Radi, R., Trujillo, M., Telleri, R., Kalyanaraman, B., Barnes, S., Kirk, M. and Freeman, B.A. (1994) J. Biol. Chem. 269, 26066–26075.
[12] Malo-Ranta, U., Yla-Herttuala, S., Metsa-Ketela, T., Jaakkola, O., Moilanen, E., Vourinen, P. and Nikkari, T. (1994) FEBS Lett. 179–183.
[13] Padmaja, S. and Huie, R.E. (1993) Biochem. Biophys. Res. Commun. 195, 539–544.
[14] Niki, E. (1990) Methods Enzymol. 186, 100–108.
[15] Haabie, J.A., Klose, J.R., Wink, D.A. and Keefer, L.K. (1993) J. Org. Chem. 58, 1472–1476.
[16] Yamamoto, Y., Niki, E., Kamiya, Y. and Shimasaki, H. (1984) Biochim. Biophys. Acta 795, 332–340.
[17] Noguchi, N., Gotoh, N. and Niki, E. (1993) Biochim. Biophys. Acta 1168, 348–357.
[18] Dee, G., Rice-Evans, C., Obeyesekera, S., Meraji, S., Jacobs, M. and Bruckdorfer, K.R. (1991) FEBS Lett. 294, 38–42.
[19] Burton, G.W., Doba, T., Gabe, E.J., Hughes, L., Lee, F.L. Prasad, L. and Ingold, K.U. (1985) J. Am. Chem. Soc. 107, 7035–7065.
[20] Niki, E., Saito, T., Kawakami, A. and Kamiya, Y. (1984) J. Biol. Chem. 259, 4177–4182.
[21] Niki, E., Takahashi, M. and Komuro, E. (1986) Chem. Lett. 1573–1576.
[22] Barclay, L.R.C., Baskin, K.A., Dakin, K.A., Locke, S.J. and Vinqvist, M.R. (1990) Can. J. Chem. 68, 2258–2268.
[23] Simic, M.G. (1991) J. Environ. Sci. Health C9, 113–153.
[24] Pryor, W.A., Tamura, M. and Church, D.F. (1984) J. Am. Chem. Soc. 106, 5073–5079.
[25] Pryor, W.A., Castle, L. and Church, D.F. (1985) J. Am. Chem. Soc. 107, 211–217.
[26] Hughes, M.N. and Nicklin, H.G. (1968) J. Chem. Soc. A 450–452.
[27] Koppenol, W.H., Moreno, J.J., Pryor, W.A., Ischiropoulos, H. and Beckman, J.S. (1992) Chem. Res. Toxicol. 5, 834–842.
[28] Huie, R.E. and Padmaja, S. (1993) Free Rad. Res. Comm. 18, 195–199.
[29] Kobayashi, K. and Miki, M. (1994) Frontiers of Reactive Oxygen Species in Biology and Medicine (K. Asada and T. Yoshikawa, eds.) Elsevier, Amsterdam, The Netherlands, pp. 223–224.