Is Nitric Oxide (NO) an Antioxidant or a Prooxidant for Lipid Peroxidation?

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Antioxidant and prooxidant effects of nitric oxide (NO) on lipid peroxidation in aqueous and non-aqueous media were examined. In an aqueous solution, NO did not induce peroxidation of unoxidized methyl linoleate (ML) and suppressed the radical initiator-induced oxidation of ML. NO suppressed the Fe(II) ion-induced oxidation of mouse liver microsomes. NO reduced the O2 consumption during the radical initiator-induced oxidation of linoleic acid in an aqueous medium. NO conversion into NO2 in an aqueous medium was not affected by unoxidized ML and was slightly reduced by peroxidizing ML. On the other hand, as well as pure NO2, NO induced peroxidation of unoxidized ML in n-hexane in a dose-dependent fashion. NO did not suppress the radical initiator-induced oxidation of ML in n-hexane. Nitrogen oxide species (NO or N2O3) formed by autoxidation was dramatically lost in n-hexane in the presence of unoxidized ML. The results indicated that NO terminated lipid peroxidation in an aqueous medium, whereas NO induced lipid peroxidation in a non-aqueous medium. Hence, NO showed both antioxidant and prooxidant effects on lipid peroxidation depending on the solvents.

Key words nitric oxide; antioxidant; prooxidant; lipid peroxidation; thiobarbituric acid-reactive substance

Nitric oxide (NO) has many important biological functions, but the NO chemistry is complex because it is converted into reactive nitrogen oxide species by contact with O2 which are in turn hydrolyzed into other nitrogen oxide species in aqueous media. NO gains reactivity when it reacts with O2 and is converted into nitrogen oxide species NO2 and N2O3 by Eqs. 1 and 2 both in aqueous and non-aqueous media. N2O3 in turn hydrolyzed into nitrite (NO2−) formation by decomposing primary lipid peroxidation has been shown, whereas adverse enhancing effect of NO on lipid peroxidation has been explained by reaction of thiols and secondary amines, respectively, with NO/O2. Guanosine is converted into xanthosine and oxosine by NO/O2.

There are many studies showing the reactivity and the decomposition of NO in relation to the biological functions. For instance, S-nitrosothiols and N-nitroamines are formed by reaction of thiols and secondary amines, respectively, with NO/O2. Guanosine is converted into xanthosine and oxosine by NO/O2.

There are conflicting observations on the effects of NO on lipid peroxidation. Protective effect of NO on lipid peroxidation has been shown, whereas adverse enhancing effect of NO on peroxidation of low density lipoprotein (LDL) has been reported. NO protects alkyl peroxide-mediated cytotoxicity, and inhibits thiobarbituric acid-reactive substance (TBARS) formation by decomposing primary lipid peroxidation products and later stage TBARS precursors. Antioxidant effect of NO on lipid peroxidation has been explained by terminating the radical chain reaction through the reaction of NO with lipid peroxo radical (ROO•) to form adducts by Eq. 5.

4NO + 2ROO• + H2O → 2ROONO + 2NO + H2O

However, adverse prooxidant effect of NO on lipid peroxidation has been suggested because NO2 that is readily produced from NO induces lipid peroxidation.

The aim of the present study was to find the environmental conditions where NO acted as an antioxidant or a prooxidant for lipid peroxidation. It was found that NO acted as an antioxidant against the radical induced lipid peroxidation in an aqueous medium, whereas NO acted as a prooxidant against unoxidized lipids in a non-aqueous medium. In an aqueous medium NO rapidly reacted with the peroxy radicals to terminate the radical chain reaction, and in a non-aqueous medium NOO3 or N2O3 generated from NO initiated the radical chain reaction of unoxidized lipids.

Experimental

Materials Purified air, NO gas (purity 99.8%) and NO2 gas (5% in nitrogen gas) were obtained from Nihonsanso Ltd., Tochigi, Japan. Nitrogen gas (purity 99.9%) was obtained from Taiyo-Sanso Ltd., Kunagawa, Japan. Methyl linoleate (ML) was obtained from Tokyo Chemical Industry, Ltd., Tokyo, Japan. Linoleic acid was obtained from Nippon Oil and Fats Company, Ltd., Tokyo, Japan. 2-Thiobarbituric acid (TBA) was purchased from Nacalai Tesque, Kyoto, Japan. 2,2’-Azobis(2-aminopropane)hydrochloride (AAPH) and 2,2’-azobis(2′,4′-dimethylvaleronitril) (AMVN) were obtained from Wako Pure Chemical Industries, Osaka, Japan.

All the aqueous solutions were prepared with deionized distilled, purified using a Milli-Q water purification system (Simipl Lab. Nihon Millipore Ltd., Tokyo, Japan), and finally by passing through a column of Chelex 100 resin (sodium form, 100—200 mesh) (Bio-Rad laboratories, CA, U.S.A.).

Mouse Liver Microsomes Mice were sacrificed by bleeding from common carotid arteries after anesthetization with chloroform. Liver was quickly isolated and washed well with cold physiological saline. Microsomes were obtained according to the method of Alb et al. A microsomal suspension in 25 ms 3-(N-morpholino)propanesulfonic acid buffer (pH 7.5) containing 0.25 m mannitol was obtained. Protein content in the microsomal suspension was determined according to the Bradford method using a Protein Assay Rapid Kit (Wako).

Nitrogen Oxide Species. NO Aqueous Solution and NO n-Hexane Solution A 100 ml NO solution in deaerated 0.1 m phosphate buffer (pH 7.4) [NO aqueous solution] or n-hexane [NO n-hexane solution] was prepared by introducing pure NO gas as described elsewhere. Three cautions were paid in order to minimize contamination of NO2. NO gas was purified by a column of KOH pellet to remove NO2 in the NO gas tank generated by dismutation of NO. A column of sodium hydrosulfite on glass wool was automatically lost in n-hexane.

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were determined to be 1.5±0.15 (S.D.) mM NO/0.01±0.00 (S.D.) mM NO2/0.1±0.01 (S.D.) mM NO3/0.25±0.01 (S.D.) mM NO3 (average of 5 determinations). The concentrations of nitrogen oxide species in NO n-hexane solution were 3.80±0.25 (S.D.) mM NO/0.51±0.05 (S.D.) mM NO2/0.07±0.01 (S.D.) mM NO3 (average of 5 determinations).

**NO-n-Hexane Solution** A 100 mM NO solution in deaerated n-hexane was prepared by introducing pure NO gas. The concentrations of nitrogen oxide species in the solution were determined to be 0.6±0.1 (S.D.) mM NO/3.5±0.15 (S.D.) mM NO2 (average of 5 determinations).

**TBARS** TBARS were determined as described elsewhere. To 0.20 ml of the sample solution or suspension, 650 μl of a mixture of 0.20 ml of 5.2% (w/v) sodium dodecyl sulfate in water, 50 μl of 0.8% (w/v) butylated hydroxytoluene in glacial acetic acid, 1.50 ml of 0.8% (w/v) TBA in water and 1.70 ml of 2 mM FeCl3 in water (or 2 mM EDTA in water), and 150 μl of 20% (w/v) acetic acid buffer (pH 3.5) were added. The mixture was kept at 5°C for 1 h and then heated at 100°C for 1 h. The mixture was extracted with 1.0 ml of n-butanol/pyridine (1:1, v/v) and absorbance of the extract at 532 nm was recorded. The amount of red pigment reflecting TBARS was determined using molecular extinction coefficient of 156000 M⁻¹ cm⁻¹.

**Dissolved O2** Dissolved O2 was measured with a Galvanic type oxygen electrode, an Able DO indicator model 1032 (Tokyo, Japan). The partial oxygen pressure of the meter was set at 0mmHg by the buffer containing 2% (w/v) sodium sulphite and set at 160mmHg by the buffer saturated with air at 25°C. In a 3.0 ml sealed cuvette attached to the electrode, 2.0 ml of a mixture of 10mM linoleic acid and 10 mM AAPH in 0.1 mM phosphate buffer (pH 7.4) containing 0.2% (w/v) cholic acid sodium salt was placed. Air in the headspace of the cuvette was replaced by nitrogen gas. The mixture was incubated for 30 min with and without addition of 20 μl of NO aqueous solution intermittently 6 times (final concentration of NO added was 100 μM). Time course of the concentration of dissolved O2 was followed.

**Determination of Nitrogen Oxide Species after Agitation with ML** The amounts of nitrogen oxide species (NO, NO2, NO3) were determined by the modified Saltzman method using an apparatus described in Fig. 2 in reference. The apparatus is composed of a series of tube A containing the sample solution (for determination of NO2), connected to a nitrogen gas tank or a purified air tank, gas absorber B (for NO determination), midget impinger C and gas absorber D (for NO determination). The apparatus and the calculation methods described in reference were employed.

Tube A was used for the reaction vessel of NO/Fe(II)/O2 and NO+/O2 with ML. For measurement of nitrogen oxide species in an aqueous medium under the anaerobic conditions, a solution of 10 mM ML in 0.1 mM phosphate buffer (pH 7.4)/0.2% (w/v) butylated hydroxytoluene and the mixture was agitated at 37°C for 2 h (Fig. 1A). Effect of NO on water-soluble azo-dye radical initiator AAPH-induced peroxidation of ML in an aqueous medium was examined. When 10 mM ML solution in phosphate buffer containing 1 mM AAPH was agitated and the mixture was incubated at 37°C for 1 h under the aerobic conditions. TBA assay was performed in the presence of EDTA as described in Experimental section. Data are expressed by mean±S.D. of triplicate experiments.

**Results**

**Effect of NO on Peroxidation of ML and Microsomes in an Aqueous Medium** Whether NO by itself can induce peroxidation of ML in an aqueous medium under the aerobic conditions was examined. It was found that NO did not increase the level of TBARS of ML in phosphate buffer incubated at 37°C for 2 h (Fig. 1A). Effect of NO on water-soluble azo-dye radical initiator AAPH-induced peroxidation of ML in the aqueous medium was examined. When 10 mM ML solution in phosphate buffer containing 1 mM AAPH was agitated at 37°C for 2 h in the presence of varying amounts of NO, TBARS formation was suppressed depending on the concentration of NO (Fig. 1B). Effect of NO on Fe(II)-induced peroxidation of mouse liver microsomes in an aqueous medium was examined. When a microsomal suspension was agitated with 0.5 mM Fe(II) ion in Tris buffer at 37°C for 1 h in the presence of NO, TBARS formation was suppressed depending on the concentration of NO (Fig. 2).

O2 consumption during AAPH-induced oxidation of linoleic acid in the presence of NO in an aqueous medium was monitored. When a mixture of 10 mM linoleic acid and 10 mM AAPH was incubated at 37°C for 30 min, O2 at 160 mmHg (270 μM) was completely lost (Fig. 3A). By contrast, when 100 μM NO at the final concentration divided into 6 portions was added to the mixture at intermittent 5 min-interval, the amount of O2 consumed was greatly suppressed (Fig. 3B). 10 mM AAPH alone consumed only a trace amount of O2 and 100 μM NO consumed about 25 μM O2, and NO together with AAPH consumed O2 to a similar extent to that consumed by NO alone (data not shown). O2 consumption in Fig. 3B may exceed over that observed in Fig. 3A if NO and the AAPH-induced lipid peroxidation consumed O2 indepen-
The results indicated that NO prevented $O_2$ consumption due to peroxidation of linoleic acid by interacting with peroxidizing linoleic acid in the aqueous medium.

**NO Conversion in an Aqueous Medium in the Presence of Unoxidized and Peroxidizing ML**

Conversion of NO in an aqueous medium after agitation with unoxidized and peroxidizing ML was examined by the modified Saltzman method.5) NO was recovered intact in the NO aqueous solution (2 m mol NO) agitated with 10 m mol unoxidized ML at 35 °C for 1 h under anaerobic conditions (Fig. 4A). Most of NO was recovered as NO$_2$ in the NO aqueous solution agitated with unoxidized ML under aerobic conditions, as in control NO aqueous solution without ML (Fig. 4B). The results indicated that unoxidized ML did not interfere with the NO conversion into NO$_2$ through autoxidation and subsequent hydrolysis.

**Effect of NO on Peroxidation of ML in $n$-Hexane**

Peroxidation of ML was induced by NO in a dose-dependent fashion in $n$-hexane under the aerobic conditions as assessed by TBARS, and the potency of NO to induce peroxidation of ML was similar to that of pure NO$_2$. As well as pure NO$_2$, NO did not prevent TBARS formation in lipid-soluble azo-dye AMVN-induced peroxidation of ML in $n$-hexane under the aerobic conditions (Fig. 7). The results indicated that NO...
Experimental section at 35 °C for 1 h. The concentrations of NO, NO₂ and NO₂ oxidized ML under the anaerobic (A) and aerobic conditions (B) in tube A described in were aqueous or non-aqueous. NO showed antioxidant activ-

Discussion

Fig. 7. Effect of NO and NO₂ on AMVN-Induced Peroxidation of ML in n-Hexane

A mixture of 2.0 mEq ML and 10 μmol NO (or NO₂) with or without 0.2 mEq AMVN in n-hexane was incubated at 37 °C for 2 h under the aerobic conditions. A 4.0 ml aliquot of the solution was withdrawn and the solvent was removed under reduced pressure and the residue was suspended in 0.20 ml of water for TBA assay in the presence of FeCl₃. Data are expressed by mean±S.D. of triplicate experiments.

Fig. 8. NO Conversion in Contact with O₂ in n-Hexane after Agitation with ML

A 4.0 mM NO n-hexane solution (1.0 ml, 4.0 μmol) was agitated with 10.0 μmol un-

in n-hexane by contact with O₂ induced peroxidation of ML and did not terminate the radical chain reaction.

NO Conversion in n-Hexane in the Presence of Unoxi-

ized and Peroxidizing ML. NO remained intact in n-

hexane solution in the absence and presence of unoxidized ML under the anaerobic conditions (Fig. 8A). When NO was treated with unoxidized ML in n-hexane under the aerobic conditions a substantial amount of NO was lost, whereas in the control medium without ML most of NO was converted into NO₂ or N₂O₃ (Fig. 8B). The result indicated that NO was converted by contact with O₂ into components other than NO₂, N₂O₃ and NO₃ by reaction with unoxidized ML in n-hexane. This was in contrast to the observation in an aqueous medium in which NO was converted by contact with O₂ into NO₂ even in the presence of unoxidized ML (Fig. 4B).

Discussion

In the present study, adverse effects of NO on lipid peroxi-
dation were observed depending on whether the solvents were aqueous or non-aqueous. NO showed antioxidant activ-

ity in an aqueous medium and prooxidant activity in n-

hexane. NO did not interact with unoxidized ML in an aque-
solution even in the presence of O₂. Through NO conver-
sion by contact with O₂ in an aqueous medium into NO₂, N₂O₃ and finally NO₂ (Eqs. 2—4) peroxidation of ML was not induced. However, NO may react with peroxidizing lipids and suppressed lipid peroxidation as assessed by TBARS for-
mation and O₂ consumption. This process may have impor-
tant significance in relation not only to biological effect of NO on lipid peroxidation but to regulatory effect of lipid per-
oxidation on biological functions of NO. Preventive effects of NO observed here on the radical initiator-induced lipid peroxidation were consistent with those of earlier studies showing the contribution of NO in the radical terminating re-
action. Protective activity of NO against lipid peroxidation has been observed in the LDL oxidation, in the superoxide and peroxynitrite-dependent lipid peroxidation, in the lipoxygenase-dependent lipid peroxidation, in the azo-dye induced lipid peroxidation of liposomal membranes and in the Fe(II) ion-induced brain lipid peroxidation and HL-
cells. NO protects against alkyl peroxide-mediated cyto-
toxicity. NO inhibits TBARS formation by decomposing primary lipid peroxidation products and later stage TBARS precursors. Kinetic study of the reaction of NO with lipid peroxy radicals (ROO·) has been done and Eq. 5 for the reaction is proposed. NO reacts with ROO· to form ROONO with k=2×10⁹ M⁻¹s⁻¹. Hence, NO has been re-
garded as an antioxidant to terminate radical chain reaction in an aqueous medium.

However, the effect of NO on lipid peroxidation was deli-
cate. NO in n-hexane was converted by contact with O₂ into NO₂ and N₂O₃ (Eqs. 1, 2) without suffering from hydrolysis. When NO was treated with unoxidized ML in n-hexane, the radical chain reaction was induced to form peroxidized lipids. This was quite similar to the case of the reaction of pure NO₂. The reactive nitrogen oxide species may interact with unoxidized fatty acid to abstract allylic hydrogen of the fatty acid and by subsequent addition of nitrogen containing molecules to initiate radical chain reactions, as has been shown in the NO₂ chemistry. The AMVN-induced per-
oxidation of ML in n-hexane could not be suppressed by NO. Hence, NO can be regarded as a prooxidant to induce lipid peroxidation in n-hexane.

Adverse effects of NO on lipid peroxidation may be due to different profiles of conversion of NO in an aqueous medium and in n-hexane by contact with O₂. Autoxidation of NO in an aqueous medium to produce NO₂ proceed by Eq. 4, and a mechanism is offered whereby NO first reacts with O₂ to form NO₂ with k=2.9×10⁹ M⁻¹s⁻¹ at 22 °C. The radical termination reaction by NO in an aqueous medium may be more rapid than NO autoxidation. On the other hand, NO is converted into NO₂ or N₂O₃ by contact with O₂ in n-hexane by Eqs. 1 and 2 without suffering from hydrolysis, and a mechanism is proposed whereby NO first reacts with O₂ with k=1.4×10⁹ M⁻¹s⁻¹ at 25 °C. While NO autoxidation in n-
hexane may be much slower than that in an aqueous medium, reactive NO₂ or N₂O₃ may not be lost in the solvent. Hence, potency of NO due to NO₂ or N₂O₃ to initiate radical chain reaction may exceed over the potency of NO to terminate the radical chain reaction in n-hexane.

In conclusion, NO showed adverse effects on lipid peroxi-
dation: preventive in an aqueous medium by terminating radical chain reaction, and stimulatory in a non-aqueous medium by initiating radical chain reaction. These different characteristics of NO may be due to whether the nitrogen oxide species produced by autoxidation of NO were hydrolyzed or not.

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References