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 O_2 consumption during the radical initiator-induced oxidation of linoleic acid in an aqueous medium. NO conversion into NO_2^- 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 NO_2, 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_2 or N_2O_3) 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 peroxidatiton 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,[1,2] but the NO chemistry is complex because it is converted into reactive nitrogen oxide species by contact with O_2 which are in turn hydrolyzed into other nitrogen oxide species in aqueous media. NO gains reactivity when it reacts with O_2 and is converted into nitrogen oxide species NO_2 and N_2O_3 by Eqs. 1 and 2 both in aqueous and non-aqueous media. N_2O_3 was in turn hydrolyzed into nitrite (NO_2^-) in aqueous media by Eq. 3.[3—5] A net reaction of NO with O_2 in aqueous media proceeds by Eq. 4.

$$2NO+O_2\rightarrow 2NO_2 \quad (1)$$

$$2NO_2+2NO\rightarrow 2N_2O_3 \quad (2)$$

$$2N_2O_3+2H_2O\rightarrow 4NO_2^-+4H^+ \quad (3)$$

$$4NO+O_2+2H_2O\rightarrow 4NO_2^-+4H^+ \quad (4)$$

There are many studies showing the reactivity and the decomposition of NO in relation to the biological functions. For instance, *S*-nitrosothiols[6,7] and *N*-nitrosamines[6,8] are formed by reaction of thiols and secondary amines, respectively, with NO/O_2. Guanosine is converted into xanthosine and oxanosine[9] by NO/O_2.

There are conflicting observations on the effects of NO on lipid peroxidation. Protective effect of NO on lipid peroxidation has been shown,[10—16] whereas adverse enhancing effect of NO on peroxidaition of low density lipoprotein (LDL) has been reported.[17] NO protects alkyl peroxide-mediated cytotoxicity,[18] and inhibit thiobarbituric acid-reactive substance (TBARS) formation by decomposing primary lipid peroxidation products and later stage TBARS precursors.[19] Antioxidant effect of NO on lipid peroxidation has been explained by terminating the radical chain reaction through the reaction of NO with lipid peroxy radical ($ROO\cdot$) to form adducts by Eq. 5.[20,21]

$$4NO+2ROO\cdot+H_2O\rightarrow 2ROONO+2NO+H_2O\rightarrow RONO_2+RONO+2HNO_2 \quad (5)$$

However, adverse prooxidant effect of NO on lipid peroxidation has been suggested because NO_2 that is readily produced from NO induces lipid perroxidation.[22—26]

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 NO_2 or N_2O_3 generated from NO initiated the radical chain reaction of unoxidized lipids.

Experimental

Materials Purified air, NO gas (purity 99.8%) and NO_2 gas (5% in nitrogen gas) were obtained from Nihonsanso Ltd., Tochigi, Japan. Nitrogen gas (purity 99.9%) was obtained from Taiyo-Sanso Ltd., Kanagawa, 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-amidinopropane)hydrochloride (AAPH) and 2,2′-zobis(2′,4′-dimethylvaleronitrile) (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 (Simpli 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 Albro *et al.*[27] A microsomal suspension in 25 mM 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 Bradfford method[28] 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.[5] Three cautions were paid in order to minimize contamination of NO_2. NO gas was purified by a column of KOH pellet to remove NO_2 in the NO gas tank generated by dismutation of NO. A column of sodium hydrosulfite on glass wool was attached to the flask in order to avoid exposure of the flask content to atmospheric O_2. Nitrogen gas was purged to remove NO in the headspace of the flask. The concentrations of nitrogen oxide species in NO aqueous solution

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were determined to be 1.8±0.15 (S.D.) mM NO/0.01±0.00 (S.D.) mM NO_2/0.1±0.01 (S.D.) mM NO_2^-/0.1±0.01 (S.D.) mM NO_3^- (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 NO_2/0.07±0.01 (S.D.) mM NO_2^- (average of 5 determinations).

NO_2 *n*-Hexane Solution A 100 ml NO_2 solution in deaerated *n*-hexane was prepared by introducing pure NO_2 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 NO_2 (average of 5 determinations).

TBARS TBARS were determined as described elsewhere.[29] 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 $FeCl_3$ in water (or 2 mM EDTA in water), and 150 μl of 20% (w/v) acetate 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 O_2 Dissolved O_2 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 0 mmHg by the buffer containing 2% (w/v) sodium sulfite and set at 160 mmHg 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 10 mM linoleic acid and 10 mM AAPH in 0.1 M 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 O_2 was followed.

Determination of Nitrogen Oxide Species after Agitation with ML The amounts of nitrogen oxide species (NO, NO_2 and NO_2^-) were determined by the modified Saltzman method using an apparatus described in Fig. 2 in reference.[5] The apparatus is composed of a series of tube A containing the sample solution (for determination of NO_2^-) connected to a nitrogen gas tank or a purified air tank, gas absorber B (for NO_2 determination), midget impinger C and gas absorber D (for NO determination. The operation methods and the calculation methods described in reference[5] were employed.

Tube A was used for the reaction vessel of NO/− O_2 and NO/+ O_2 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 M phosphate buffer (pH 7.4)/0.2% cholic acid sodium salt (1.0 ml) was placed in tube A and nitrogen gas was introduced for 2 h. After nitrogen gas flow was stopped, 1.0 ml of NO aqueous solution (2.0 mM) with and without 0.1 ml of deairated 10 mM AAPH solution was introduced using a gas-tight micosyringe, and the mixture was agitated at 35 °C for 1 h under the anaerobic conditions. The concentrations of NO, NO_2 and NO_2^- in the solution were determined after purging nitrogen gas.

For measurement of nitrogen oxide species in an aqueous medium under the aerobic conditions, ML solution and NO aqueous solution with or without AAPH were mixed and agitated under the aerobic conditions, and the concentrations of the nitrogen oxide species were determined after purging purified air.

For measurement of nitrogen oxide species in *n*-hexane under the anaerobic conditions, 1.0 ml of 10 mM ML in *n*-hexane was introduced in tube A, and the solvent was removed by nitrogen gas flow for 2 h. NO *n*-hexane solution (4.0 mM, 1.0 ml) was introduced using a gas-tight microsyringe, and the mixture was agitated at 35 °C for 1 h under the anaerobic conditions. The concentrations of nitrogen species in the solution were determined after purging nitrogen gas.

For measurement of nitrogen oxide species in *n*-hexane under the aerobic conditions, a solution of 1 μmol ML in NO *n*-hexane solution (4 mM, 1.0 ml) was agitated under the aerobic conditions, and the concentrations of the nitrogen oxide species were determined after purging purified air.

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).

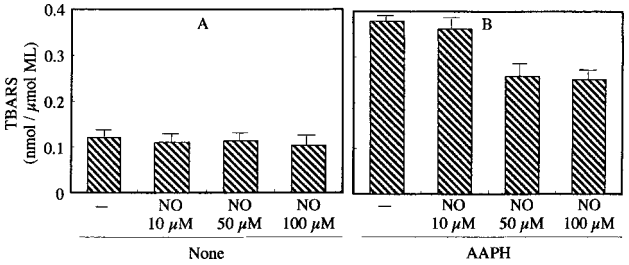

Fig. 1. Effect of NO on Peroxidation of ML (A) and on AAPH-Induced Peroxidation of ML in an Aqueous Medium

To 3.0 ml of a mixture of 10 mM ML without (A) and with (B) 1 mM AAPH in 0.1 M phosphate buffer (pH 7.4)/0.2% (w/v) cholic acid sodium salt, an aliquot of NO aqueous solution corresponding to the final concentration at 10, 50 or 100 μM NO was introduced and the mixture was incubated at 37 °C for 2 h under the aerobic conditions. TBA assay was performed in the presence of $FeCl_3$ as described in Experimental section. Data are expressed by mean±S.D. of triplicate experiments.

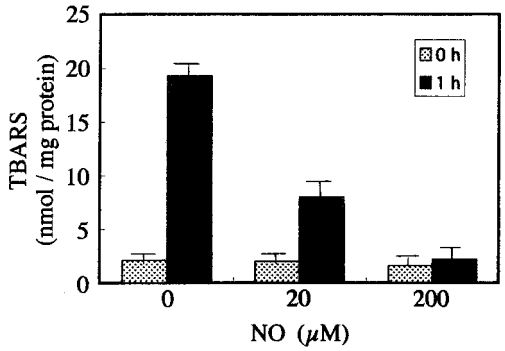

Fig. 2. Effect of NO on Fe(II) ion-Induced Peroxidation of Microsomes

To 10 ml of a microsomal suspension (1 mg protein/ml) and 0.5 mM $FeSO_4$/8.5 mM ADP in in 0.15 M KCl/50 mM Tris–HCl buffer (pH 7.4), an aliquot of NO aqueous solution corresponding to the final concentration at 10, 20 or 200 μM NO was introduced 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.

O_2 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, O_2 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 O_2 consumed was greatly suppressed (Fig. 3B). 10 mM AAPH alone consumed only a trace amount of O_2, and 100 μM NO consumed about 25 μM O_2, and NO together with AAPH consumed O_2 to a similar extent to that consumed by NO alone (data not shown). O_2 consumption in Fig. 3B may exceed over that observed in Fig. 3A if NO and the AAPH-induced lipid peroxidation consumed O_2 indepen-

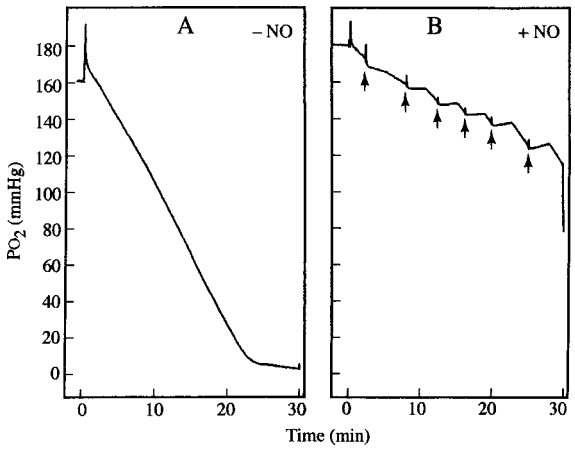

Fig. 3. Effect of NO on O_2 Consumption during the AAPH-Induced Peroxidation of Linoleic Acid in an Aqueous Medium

In a 3.0 ml sealed cuvette attached to the electrode, 2.0 ml of a mixture of 10 mM linoleic acid and 10 mM AAPH in 0.1 M phosphate buffer (pH 7.4)/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 without (A) and with addition of 20 μl of NO aqueous solution intermittently 6 times (final concentration of NO introduced: 100 μM) (B).

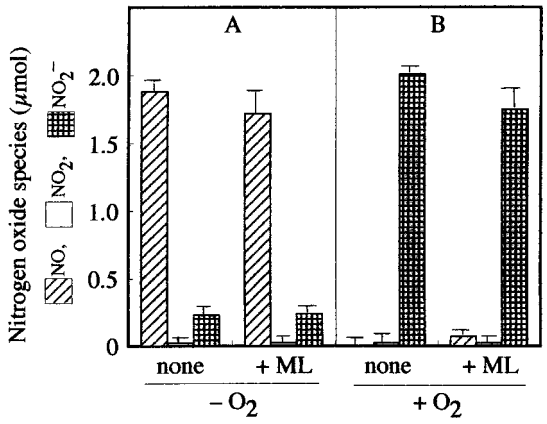

Fig. 4. NO Conversion in Contact with O_2 in an Aqueous Medium after Agitation with ML

A 1.0 mM NO aqueous solution (2.0 ml, 2.0 μmol) was agitated with 10.0 μmol unoxidized ML in 0.1 M phosphate buffer (pH 7.4)/0.2% cholic acid sodium salt under the anaerobic (A) and aerobic conditions (B) in tube A described in Experimental section at 35 °C for 1 h. The concentrations of NO, NO_2 and NO_2^- in the solution were determined by the modified Saltzman method. Data are expressed by mean±S.D. of triplicate experiments.

dently. 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 μmol NO) agitated with 10 μmol unoxidized ML at 35 °C for 1 h under the anaerobic conditions (Fig. 4A). Most of NO was recovered as NO_2^- in the NO aqueous solution agitated with unoxidized ML under the 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.

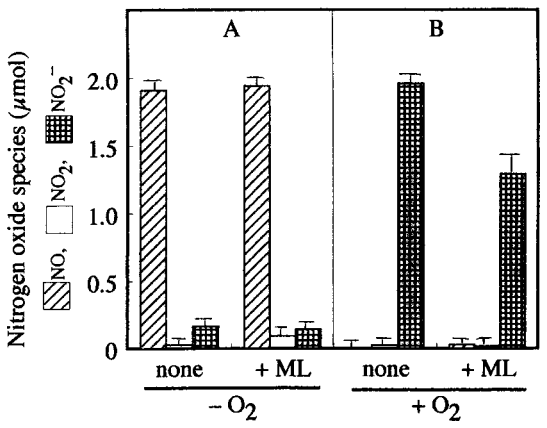

Fig. 5. NO Conversion in Contact with O_2 during the AAPH-Induced Oxidation of ML in an Aqueous Medium

A 1.0 mM NO aqueous solution (2.0 ml, 2.0 μmol) was agitated with 10.0 μmol unoxidized ML and 1.0 μmol AAPH in 0.1 M phosphate buffer (pH 7.4)/0.2% cholic acid sodium salt under the anaerobic (A) and aerobic conditions (B) in tube A described in Experimental section at 35 °C for 1 h. The concentrations of NO, NO_2 and NO_2^- in the solution were determined by the modified Saltzman method. Data are expressed by mean±S.D. of triplicate experiments.

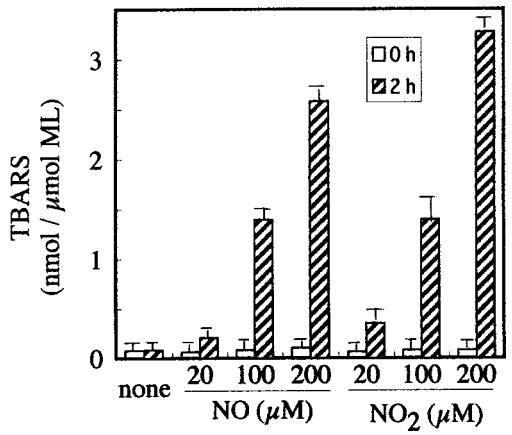

Fig. 6. Effect of NO and NO_2 on Perxidation of in *n*-Hexane

A mixture of 2.0 mM ML and NO or NO_2 at the indicated concentration 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 $FeCl_3$. Data are expressed by mean±S.D. of triplicate experiments.

Most of NO was recovered intact in the NO aqueous solution agitated with unoxidized ML in the presence of AAPH under the anaerobic conditions (Fig. 5A). Recovery of NO was significantly reduced in the NO aqueous solution agitated with ML peroxidizing with AAPH under aerobic conditions (Fig. 5B). The result may indicate that NO was trapped by peroxidizing ML to produce components that would not release NO_2^- (Eq. 5) during the assay. This NO consumption may reflect the observation showing that increase in TBARS in the AAPH-induced oxidation of ML was suppressed by NO (Fig. 1).

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

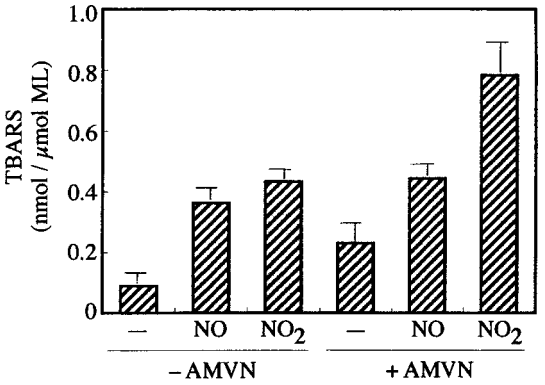

Fig. 7. Effect of NO and NO_2 on AMVN-Induced Peroxidation of ML in *n*-Hexane

A mixture of 2.0 mM ML and 10 μM NO (or NO_2) with or without 0.2 mM 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 $FeCl_3$. Data are expressed by mean±S.D. of triplicate experiments.

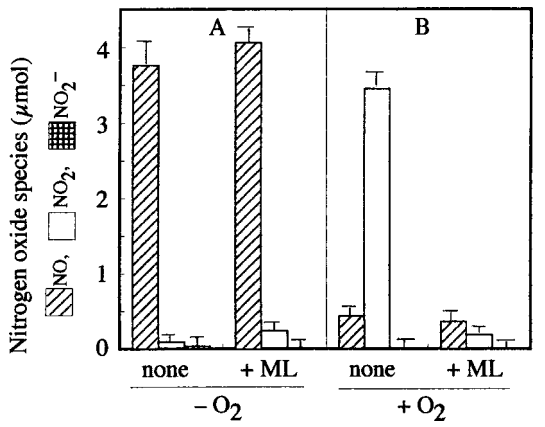

Fig. 8. NO Conversion in Contact with O_2 in *n*-Hexane after Agitation with ML

A 4.0 mM NO *n*-hexane solution (1.0 ml, 4.0 μmol) was agitated with 10.0 μmol unoxidized ML under the anaerobic (A) and aerobic conditions (B) in tube A described in Experimental section at 35 °C for 1 h. The concentrations of NO, NO_2 and NO_2^- in the solution were determined by the modified Saltzman method. Data are expressed by mean±S.D. of triplicate experiments.

in *n*-hexane by contact with O_2 induced peroxidation of ML and did not terminate the radical chain reaction.

NO Conversion in *n*-Hexane in the Presence of Unoxidized 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 NO_2 or N_2O_3 (Fig. 8B). The result indicated that NO was converted by contact with O_2 into components other than NO_2 N_2O_3 and NO_2^- 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 O_2 into NO_2^- even in the presence of unoxidized ML (Fig. 4B).

Discussion

In the present study, adverse effects of NO on lipid peroxidation were observed depending on whether the solvents were aqueous or non-aqueous. NO showed antioxidant activity in an aqueous medium and prooxidant activity in *n*-hexane. NO did not interact with unoxidized ML in an aqueous solution even in the presence of O_2. Through NO conversion by contact with O_2 in an aqueous medium into NO_2, N_2O_3 and finally NO_2^- (Eqs. 2—4) peroxidation of ML was not induced. However, NO may react with peroxidizing lipids and suppressed lipid peroxidation as assessed by TBARS formation and O_2 consumption. This process may have important significance in relation not only to biological effect of NO on lipid peroxidation but to regulatory effect of lipid peroxidation 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 reaction. Protective activity of NO against lipid peroxidation has been observed in the LDL oxidation,[10] in the superoxide and peroxynitrite-dependent lipid peroxidation,[11,12] in the lipoxygenase-dependent lipid peroxidation,[13] in the azo-dye induced lipid peroxidation of liposomal membranes[14] and in the Fe(II) ion-induced brain lipid peroxidation[15] and HL-cells.[16] NO protects against alkyl peroxide-mediated cytotoxicity.[18] NO inhibits TBARS formation by decomposing primary lipid peroxidation products and later stage TBARS precursors.[19] Kinetic study of the reaction of NO with lipid peroxy radicals (ROO·) has been done and Eq. 5 for the reaction is proposed.[20,21] NO reacts with ROO· to form ROONO with $k=2\times10^9\,\mathrm{M^{-1}\,s^{-1}}$.[20] Hence, NO has been regarded as an antioxidant to terminate radical chain reaction in an aqueous medium.

However, the effect of NO on lipid peroxidation was delicate. NO in *n*-hexane was converted by contact with O_2 into NO_2 and N_2O_3 (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 NO_2. 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 NO_2 chemistry.[22—26] The AMVN-induced peroxidation 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 O_2. Autoxidation of NO in an aqueous medium to produce NO_2^- proceeds by Eq. 4, and a mechanism is offered whereby NO first reacts with O_2 to form NO_2 with $k=2.9\times10^6\,\mathrm{M^{-2}\,s^{-1}}$ at 22 °C.[6] The radical termination reaction by NO in an aqueous medium may be more rapid than NO autoxidaiton. On the other hand, NO is converted into NO_2 or N_2O_3 by contact with O_2 in *n*-hexane by Eqs. 1 and 2 without suffering from hydrolysis, and a mechanism is proposed whereby NO first reacts with O_2 with $k=1.4\times10^4\,\mathrm{M^{-2}\,s^{-1}}$ at 25 °C.[30] While NO autoxidation in *n*-hexane may be much slower than that in an aqueous medium, reactive NO_2 or N_2O_3 may not be lost in the solvent. Hence, potency of NO due to NO_2 or N_2O_3 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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