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# Nitric Oxide Inhibits Iron–Induced Lipid Peroxidation in HL-60 Cells[1]

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**Nitric oxide (˙NO) can protect cells against the detrimental effects of reactive oxygen species. Using low-density lipoprotein as well as model systems, it has been demonstrated that ˙NO can serve as a chain-breaking antioxidant to blunt lipid peroxidation. To test the hypothesis that ˙NO can serve as a chain-breaking antioxidant in cell membranes, we examined the effect of ˙NO on iron-induced lipid peroxidation in human leukemia cells. We exposed HL-60 cells to an oxidative stress (20 $\mu$M $Fe^{2+}$) and monitored the consumption of oxygen as a measure of lipid peroxidation. Oxygen consumption was arrested by the addition of ˙NO as a saturated aqueous solution. The duration of inhibition of oxygen consumption by ˙NO was concentration-dependent in the 0.4–1.8 $\mu$M range. The inhibition ended upon depletion of ˙NO. The addition of ˙NO prior to initiation of peroxidation delayed the onset of peroxidation; the nearer in time it was before $Fe^{2+}$ addition, the longer the inhibition. Depletion of cellular glutathione levels by D,L-buthionine-*S*,*R*-sulfoximine prior to $Fe^{2+}$ addition resulted in a more rapid initial rate of oxygen depletion and a shorter time for the ˙NO-induced inhibition of oxygen consumption. Complementary studies of this iron-induced lipid peroxidation, using thiobarbituric acid reactive substances as a marker, also demonstrated the protective effects of ˙NO. This protection of cells against lipid peroxidation also manifested itself as a reduction in trypan blue uptake, an observation demonstrating the protective effects of ˙NO on membrane integrity. We conclude that ˙NO protects HL-60 human leukemia cells from lipid peroxidation and that this protection ameliorates the toxicity of the oxidation processes initiated by $Fe^{2+}$ and dioxygen.** © 1999 Academic Press

*Key Words:* **nitric oxide; lipid peroxidation; oxygen consumption; iron, free radicals.**

There is great interest in the role of nitric oxide (˙NO) in biology because it can be a signaling molecule, a toxin, a prooxidant, and a potential antioxidant. It is involved in signaling in vasodilatation (1–4) and neurotransmission (4, 5), a toxin in the destruction of pathogens (4), and an oxidant (6). However, its diverse chemistry and its biologic activity sometimes are seemingly contradictory. Nowhere is this contradiction more evident than in oxidative stress (7, 8). Nitric oxide has been proposed to act as a prooxidant when it reacts with superoxide ($O_2^{\cdot -}$), forming the highly reactive peroxynitrite ($O{=}NOO^-$) (6, 9). On the other hand, ˙NO can also inhibit oxidation. Nitric oxide can cause chain termination reactions during lipid peroxidation, as observed in low-density lipoprotein oxidation as well as in chemical systems (10–13).

Lipid peroxidation has three major components: initiation, propagation, and termination.

$$LH + \text{oxidant}^{\cdot} \rightarrow L^{\cdot} + \text{oxidant-H} \quad \text{(initiation)} \quad [1]$$

$$L^{\cdot} + O_2 \rightarrow LOO^{\cdot} \quad \text{(propagation cycle)} \quad [2]$$

$$LOO^{\cdot} + LH \rightarrow L^{\cdot} + LOOH \quad \text{(propagation cycle)} \quad [3]$$

$$L^{\cdot} + L^{\cdot} \rightarrow \text{non-radical products} \quad \text{(termination)} \quad [4]$$

$$L^{\cdot} + LOO^{\cdot} \rightarrow \text{non-radical products} \quad \text{(termination)} \quad [5]$$

It is in the termination process that ˙NO has been proposed to play a crucial role as an antioxidant in lipid

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peroxidation (10). O'Donnell *et al.* have studied the reaction of ˙NO with lipid-derived radicals (13). Using 2,2′-azobis(2-amidinopropane) hydrochloride as an initiator to study the peroxidation of linoleate in cell-free model systems, they found that 0.5–20 $\mu$M ˙NO caused inhibition of $O_2$ consumption that continued until depletion of the ˙NO. Based on their experimental results and kinetic considerations, they proposed that ˙NO could serve as an antioxidant by intercepting chain-carrying peroxyl radicals,

$$LOO^{\cdot} + {}^{\cdot}NO \rightarrow LOONO \quad \text{(chain termination)} \quad [6]$$

This chain-termination reaction has the same result as that of typical donor antioxidants such as vitamin E in that the chain carrying peroxyl radical is removed.

$$LOO^{\cdot} + \text{antioxidant-H} \rightarrow LOOH + \text{antioxidant}^{\cdot} \quad \text{(chain termination)} \quad [7]$$

However, it is clear that different pathways will be involved in the removal of the antioxidant radical of reaction [7] and the LOONO formed in reaction [6].

Nitric oxide has been shown to protect against the cytotoxicity of oxidative stress in endothelial cells, fibroblasts, hepatocytes, intestinal epithelium, and cardiomyocytes (14–20). However, there is much yet to be learned about the molecular mechanisms of this protection and whether ˙NO can inhibit Fenton-mediated oxidative reactions in live intact cells and thereby protect them against oxidative stress. To address the hypothesis that ˙NO is an important membrane antioxidant, we have studied the effect of ˙NO on $Fe^{2+}$-mediated lipid peroxidation in intact, functional HL-60 cells.

## EXPERIMENTAL PROCEDURES

*Cell culture and fatty acid modification.* HL-60 human leukemic cells were obtained from American Type Culture Collection and grown in RPMI 1640 containing 10 $\mu$M L-glutamine and 10% fetal bovine serum (FBS)[3] at 37°C, 5% $CO_2$/95% air. For fatty acid modification, cells were seeded in the above growth medium supplemented with 32 $\mu$M docosahexaenoic acid (22:6$\omega$3) and grown for 48 h. Cells were then harvested, centrifuged, and resuspended in normal saline at $5 \times 10^6$ cells/ml for $O_2$ consumption experiments. The extent of fatty acid modification by gas chromatography has been previously described (21, 22). The studies described here were performed using HL-60 cells that had been grown in the presence of the highly polyunsaturated fatty acid docosohexaenoic acid in order to enhance oxidizability (23) and make the measurements of lipid peroxidation inhibition more sensitive. However, studies with unmodified cells revealed the same pattern of inhibition by ˙NO, but all measurements were more difficult and the effect was less marked as would be expected. Membrane integrity studies were conducted using trypan blue dye exclusion.

*Nitric oxide preparation and measurement.* Aqueous ˙NO solutions were prepared by mixing 2 parts 1 M $NaNO_2$ with 1 part 1 M HCl in an $O_2$-free, gas-tight bottle. The ˙NO gas liberated from this reaction was bubbled through oxygen-free distilled $H_2O$ (24). This process was allowed to continue for 2 h before the solution was used for experimentation. Oxygen purging was accomplished using $N_2$. A saturated ˙NO solution under our experimental conditions is about 1.8 mM. ˙NO concentrations were determined using a Sievers Model 280 Nitric Oxide Analyzer (Boulder, CO).

*Oxygen consumption.* For $O_2$ uptake studies the cell suspension (4.0 ml) at 25°C was added to a YSI Model 5300 Oxygen Monitor (Yellow Springs, OH) (25). After establishing a baseline rate of $O_2$ consumption, peroxidation was initiated by the addition of 20 $\mu$M ferrous sulfate. The duration of the inhibition of lipid peroxidation by ˙NO was determined by drawing three lines on the chart paper that recorded the $O_2$ consumption. One line was a tangent representing the slope before ˙NO introduction, the second a tangent to the slope during the inhibition period, and the third a tangent representing the slope upon the resumption of rapid $O_2$ uptake. The two intersections provided the duration of inhibition.

Nitric oxide can be an interfering gas for the YSI Model 5300 Biological Oxygen monitor in that with a saturated aqueous solution of ˙NO, the electrode has about one-third the response given by $O_2$.[4] In the experiments reported here [˙NO] $\leq$ 2 $\mu$M. Therefore, any response of the electrode to ˙NO in these experiments is below the limit of detection, and thus ˙NO is not an interfering gas for $O_2$ measurement in our experiments.

*Thiobarbituric acid reactive substances.* Cells were treated in the $O_2$ monitor as described above and then aliquots of $5 \times 10^6$ cells were taken just prior to $Fe^{2+}$ addition and 5 min after the $Fe^{2+}$ addition. In experiments that included ˙NO, it was added 1 min after the $Fe^{2+}$ addition. Samples were analyzed using the 2-thiobarbituric acid assay (26, 27) using 1,1,3,3-tetramethoxypropane hydrolyzed in trichloroacetic acid as a standard.

*Iron determination.* Iron as $Fe^{2+}$ was determined using Ferrozine, $\epsilon_{560}$ = 27,900 $M^{-1}cm^{-1}$ (28). To 100 $\mu$l of an 8 mM aqueous Ferrozine stock solution (in 100 mM sodium acetate) was added the solution containing $Fe^{2+}$ with rapid mixing. Then water was added so that the final volume was 1 ml. The absorbance at 560 nm was then determined. The blank contained all additions except $Fe^{2+}$. When cell suspensions were analyzed, the cells were removed by centrifugation after the addition of all reagents.

## RESULTS AND DISCUSSION

### *Nitric Oxide Inhibits $Fe^{2+}$-Induced Lipid Peroxidation*

Oxidative stress in cells can be initiated by the addition of $Fe^{2+}$ in the presence of dioxygen. This stress will result in lipid peroxidation and the associated formation of lipid radicals (29, 30). These carbon-centered lipid radicals react with $O_2$, resulting in consumption of $O_2$ above that from normal respiration. The rate of loss of $O_2$ will be proportional to the rate of lipid peroxidation in the cell.

Since ˙NO can inhibit lipid peroxidation in chemical and biochemical systems, we tested whether ˙NO can

[3] Abbreviations used: BSO, D,L-buthionine-*S*,*R*-sulfoximine; FBS, fetal bovine serum; GPx, glutathione peroxidase; L-NAME, $N^{\omega}$-nitro-L-arginine-methyl ester; PhGPx, phospholipid hydroperoxide glutathione peroxidase; PUFA, polyunsaturated fatty acids; SNAP, *S*-nitroso-*N*-acetyl-D,L-penicillamine; TBARS, thiobarbituric acid reactive substances; TOH, vitamin E.

[4] YSI Model 5300 Biological Oxygen Monitor Instruction Manual, Scientific Division, YSI Instrument Co., Inc.

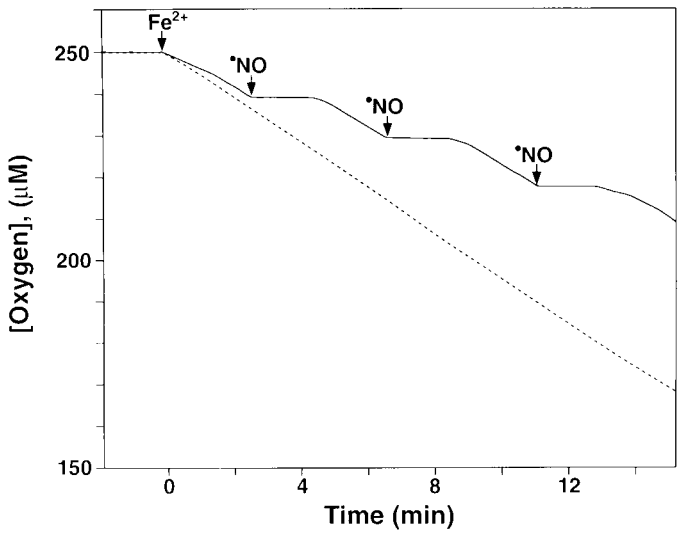


**FIG. 1.** ˙NO inhibits iron-induced lipid peroxidation. The rate of $O_2$ consumption of HL-60 cells ($5 \times 10^6$/ml and 4 ml) was determined using a YSI oxygen monitor. $Fe^{2+}$ (20 $\mu$M) was added at the first arrow and subsequently ˙NO (1.8 $\mu$M) was added (other arrows). When ˙NO was added the $O_2$ consumption was inhibited for a period of a few minutes and then it resumed at near its initial rate until the reintroduction of additional ˙NO. Also shown (lower dashed line) is a control of HL-60 cells subjected to $Fe^{2+}$-induced oxidative stress in the absence of ˙NO addition. The background rate of oxygen uptake of the HL-60 cell suspension before the addition of $Fe^{2+}$ was 10 nM/s. Upon the addition of 20 $\mu$M $Fe^{2+}$, this rate increased to 220 nM/s. The addition of ˙NO resulted in a decrease in $O_2$ consumption to $<$10 nM/s.

inhibit lipid peroxidation in live intact cells. We observed that low micromolar concentrations ($<2$ $\mu$M) of ˙NO inhibited $Fe^{2+}$-induced $O_2$ consumption in HL-60 cells (Fig. 1). These observations are consistent with other work with low micromolar concentrations of ˙NO in purified lipid suspensions (13).

After a few minutes, the rate of $O_2$ consumption returned to approximately its original rate. It seemed likely that this resumption of rapid $O_2$ consumption is due to the depletion of ˙NO since subsequent ˙NO additions each inhibited $O_2$ consumption with kinetics similar to the first addition (Fig. 1). It order to demonstrate this, we determined the [˙NO] at selected times during the inhibition. Peroxidation was initiated with 20 $\mu$M $Fe^{2+}$; at 1 min after the addition of $Fe^{2+}$, 0.9 $\mu$M ˙NO was added. As seen in Fig. 2, ˙NO was rapidly depleted and was below the limit of detection at about 4 min, after which time $O_2$ consumption resumed. This reinstitution of $O_2$ consumption at 4 min is likely due to residual $Fe^{2+}$, which is approximately 7.2 $\mu$M (Fig. 2). We have observed in separate experiments that under the conditions used in our experiments, detectable $O_2$ consumption can be initiated by 10 and 5 $\mu$M ferrous iron, but not by lower concentrations such as 1.5 $\mu$M (data not shown). Thus, we believe that the reinitiation of peroxidation after the depletion of ˙NO is most likely due to the remaining ferrous iron present.

Several types of control experiments were performed. The addition of an aliquot of nitrogen-saturated water rather than the ˙NO solution had no effect on peroxidation, indicating that the observed results are dependent upon the presence of ˙NO. The addition of L-arginine (100 $\mu$M), a biological precursor of ˙NO, did not affect the inhibition, suggesting that cellular synthesis of ˙NO is not involved. Last, when L-NAME (100 $\mu$M), which inhibits cellular production of ˙NO, was added before the initiation of peroxidation by $Fe^{2+}$, there was no effect on $O_2$ consumption. This demonstrates that cellular nitric oxide synthase was not an active participant in contributing to the inhibition of $O_2$ consumption we observed.

A logical question that arises from these experiments is whether ˙NO inhibits the participation of $Fe^{2+}$ in these processes. It has been demonstrated that the reaction of ˙NO with heme and nonheme iron can prevent the formation of oxoferryl-Hb-derived species in the presence of peroxides; these species damage cells (31, 32). However, to provide maximum protection, [˙NO] must be greater than [iron species]. Although ˙NO and iron have a rich shared chemistry, we rule this out as being a primary factor in the observed inhibition of lipid peroxidation. At the time of introduction of 0.9 $\mu$M ˙NO, the $Fe^{2+}$ concentration is $\geq$7.2 $\mu$M; yielding an [$Fe^{2+}$] to [˙NO] ratio of about 8:1. However,

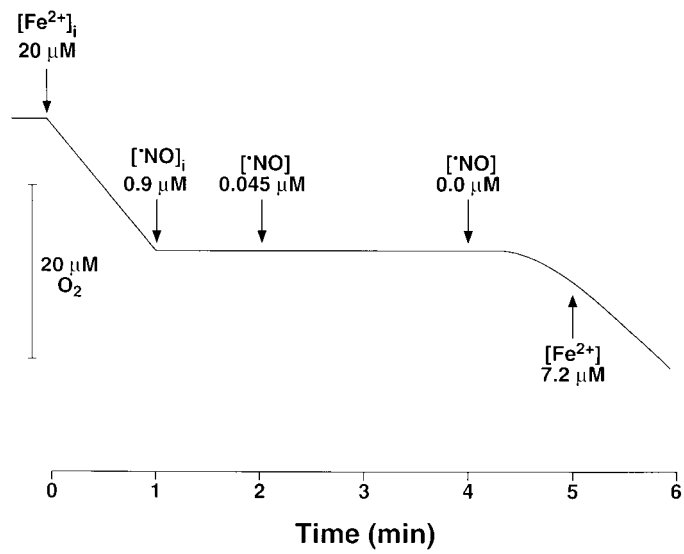


**FIG. 2.** Changes in concentration of ˙NO and $Fe^{2+}$ during cellular lipid peroxidation and its inhibition by ˙NO. Shown are the concentrations of ˙NO and $Fe^{2+}$ at key time points. Peroxidation was initiated with 20 $\mu$M $Fe^{2+}$. At 1 min after the addition of $Fe^{2+}$, 0.9 $\mu$M ˙NO was introduced. ˙NO was rapidly depleted and is below the limit of detection at about 4 min. At the time of ˙NO depletion, rapid $O_2$ uptake resumes. This reinitiation of $O_2$ consumption is due to $Fe^{2+}$ that is still present at 7.2 $\mu$M or about 36% of its original value.

at the 2-min time point, this ratio is greater than 150:1. Because $Fe^{2+}$ is in such great excess, any chemistry that involves $Fe^{2+}$ and $^{\cdot}NO$ would have $^{\cdot}NO$ as the limiting reagent. With so little $^{\cdot}NO$ present, inhibition of $Fe^{2+}$ chemistry by traditional stoichiometric processes is not likely, suggesting that $^{\cdot}NO$ is serving as a chain-breaking antioxidant during cellular lipid peroxidation.

### *Nitric Oxide Inhibition of Lipid Peroxidation Is Concentration Dependent*

The magnitude of the $^{\cdot}NO$ effect can be quantitated by measuring the duration of inhibition of $O_2$ consumption. We observed that when $^{\cdot}NO$ (0–1.8 $\mu$M) was added to the cell suspension after the initiation of lipid peroxidation by $Fe^{2+}$, there was an approximately linear increase in the duration of inhibition of lipid peroxidation vs [$^{\cdot}NO$] (Fig. 3, top). Concentrations of $^{\cdot}NO$ above approximately 2 $\mu$M produced a very rapid consumption of $O_2$ in the presence and or absence of cells (Fig. 3, top, inset). We attribute these higher rates of $O_2$ consumption to the reaction of $^{\cdot}NO$ with dioxygen: $4\,^{\cdot}NO + O_2 + 2H_2O \rightarrow 4\,NO_2^- + 4H^+$ (33, 34) as well as the anaerobic dilution of the sample. In addition, the estimated biological concentration of $^{\cdot}NO$ is typically in the range of 10–400 nM (35). Thus, the use of low levels of $^{\cdot}NO$ better mimic its biological concentrations, and the results obtained would better represent its *in vivo* action.

### *Addition of $^{\cdot}NO$ Prior to $Fe^{2+}$ also Inhibits Peroxidation*

When $^{\cdot}NO$ was added before the initiation of peroxidation by $Fe^{2+}$, there was a time-dependent delay in the initiation of peroxidation (Fig. 3, bottom). This delay in the onset of peroxidation was greater for $^{\cdot}NO$ additions closest in time prior to the addition of $Fe^{2+}$ and were much less for $^{\cdot}NO$ added 2–3 min before the addition of $Fe^{2+}$. This is consistent with the loss of $^{\cdot}NO$ via chemical processes, such as the conversion to nitrite (vida supra). This loss of $^{\cdot}NO$ would shorten the time of inhibition. This effect of time-of-addition of $^{\cdot}NO$ prior to introducing $Fe^{2+}$ is in contrast to the situation when the $Fe^{2+}$ is added first, in which case there is no effect of interval between the two additions. Regardless of the cause for maximum inhibition of peroxidation, the data presented in Fig. 3 demonstrate that the $^{\cdot}NO$ must be added immediately before the initiating event or after the event to maximize the protection by $^{\cdot}NO$. These observations rule out the involvement of stable products of $^{\cdot}NO + O_2$ chemistry as being important in the inhibition of lipid peroxidation and clearly point to $^{\cdot}NO$ as the species responsible for the inhibition of $O_2$ consumption.

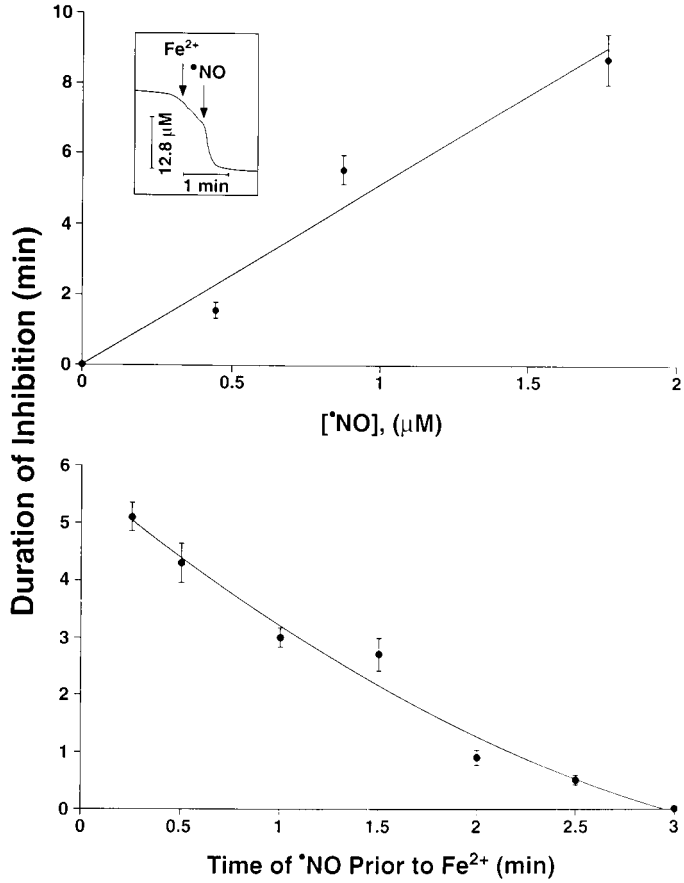


**FIG. 3.** $^{\cdot}NO$ inhibits lipid peroxidation in a concentration-dependent manner and addition of $^{\cdot}NO$ prior to the addition of iron inhibits lipid peroxidation. (Top) Peroxidation of HL-60 cells was initiated with $Fe^{2+}$ and dioxygen, and $^{\cdot}NO$ was subsequently added at the concentrations shown. The time from the start of inhibition to resumption of the near-original rate of peroxidation, as a measure of extent of inhibition, was recorded. Each data point represents the mean of at least three independent determinations. A linear least squares line fit of the data yields a correlation coefficient of −0.98. (Inset) The effect of concentrations of $^{\cdot}NO$ higher than 1.8 $\mu$M on $O_2$ consumption. There was instantaneous $O_2$ consumption after the addition of 8.9 $\mu$M of $^{\cdot}NO$. We attribute this to the direct reaction of $^{\cdot}NO$ with $O_2$. A similar rapid loss of oxygen also occurs in the absence of cells. (Bottom) $^{\cdot}NO$ (0.9 $\mu$M) inhibits lipid peroxidation when added prior to $Fe^{2+}$ addition, and the effects diminish as the time prior to introduction of $Fe^{2+}$ increases. Each data point represents the mean of at least three independent determinations and the bars the standard error.

### *Nitric Oxide Inhibits TBARS Production*

To confirm that the effects of $^{\cdot}NO$ on $O_2$ consumption are due principally to modulation of lipid peroxidation, we examined the effect of $^{\cdot}NO$ on the production of TBARS. Cells were subjected to iron-induced peroxidation at time ($t$) = 0 and collected for the assay of TBARS at $t$ = 5 min (Table I). We observed an approximate 63% reduction in $Fe^{2+}$-induced TBARS production when $^{\cdot}NO$ was introduced (0.9 $\mu$M $^{\cdot}NO$ was added

**TABLE I**

˙NO Inhibits Production of TBARS

| Additions | TBARS (nmol/mg) | Inhibition of TBARS by ˙NO (%) | $[O_2]$ ($\mu$M) | Inhibition of oxygen uptake by ˙NO (%) |
|---|---|---|---|---|
| None (control) | 0.65 ± 0.05 | | 250 | |
| $Fe^{2+}$ | 2.65 ± 0.17 | | 183[a] | |
| $Fe^{2+}$ + ˙NO | 1.39 ± 0.15 | 63[b] | 235[a] | 78 |

*Note.* HL-60 cells ($5 \times 10^6$/ml) were placed in the oxygen monitor to reproduce the experimental conditions of Fig. 2. At the designated time points, butylated hydroxytoluene was added and samples were quick frozen for later determination of TBARS. Samples were taken at $t$ = 5 min postinitiation with $Fe^{2+}$. ˙NO (0.9 $\mu$M) was added at $t$ = 1 min. Control cells were collected for the assay of TBARS immediately prior to initiation of peroxidation with iron. The $Fe^{2+}$ and $Fe^{2+}$ + ˙NO values represent means and standard errors of five independent determinations and the control samples are the mean and standard error of three independent determinations. TBARS are expressed per milligram of protein. For comparison, the table also shows the $O_2$ concentrations from the experiments of Fig. 2. We conclude that ˙NO reduces TBARS, and the percentage of inhibition is similar to the percentage of inhibition of $O_2$ consumption.

[a] Concentration of $O_2$ at 5 min.

[b] The inhibition of TBARS represents the change after subtracting the controls.

at $t$ = 1 min after iron addition). This effect of ˙NO in the TBARS assay clearly demonstrates that lipid peroxidation is being attenuated.

This 63% reduction in TBARS is approximately what would be predicted from a comparison to the $O_2$ consumed at 1 and 5 min as in Fig. 2. When 20 $\mu$M $Fe^{2+}$ is added to the HL-60 cell suspension to initiate lipid peroxidation, $[O_2]$ decreases from 250 to 235 $\mu$M in the first min before the addition of ˙NO. If sufficient ˙NO is provided at the $t$ = 1 min point, then no more $O_2$ is consumed until after 5 min and the total consumption during the 5 min results in a decrease in $[O_2]$ by 15 $\mu$M. On the other hand, if ˙NO is not introduced into the cell suspension, $[O_2]$ is decreased by 67 $\mu$M (from 250 to 183 $\mu$M). Thus, in our experimental protocol, ˙NO decreases total $O_2$ consumption by 78% and this compares favorably with the 63% decrease in TBARS (Table I). These results confirm the link between $O_2$ consumption and TBARS production in these experiments and demonstrate that ˙NO blunts lipid peroxidation in HL-60 membranes.

### *BSO Reduces the Ability of ˙NO to Inhibit Lipid Peroxidation*

BSO decreases the total reducing potential of cells by inhibiting the production of glutathione. Glutathione is important in the reduction of cellular peroxides, including lipid peroxides. We hypothesized that if we were to reduce the cellular glutathione level of the HL-60 cells prior to initiating lipid peroxidation that there could be a larger pool of preexisting lipid hydroperoxides in cell membranes. Then, upon initiation of peroxidation, there would be more substrate for the initiator ($Fe^{2+}$) to react with and thus more oxidative reaction chains would be initiated. Therefore, cellular lipid peroxidation propagation would proceed at a faster rate and thereby produce more lipid and lipid peroxyl radicals per unit time. If this were the case then we would expect that the ˙NO would be consumed faster and the subsequent duration of inhibition would be shorter. When cells were incubated with 100 $\mu$M BSO for 24 h prior to $Fe^{2+}$ addition, the duration of inhibition was significantly reduced compared to that in experiments done without BSO (Fig. 4). This is consistent with our hypothesis that the lowering of

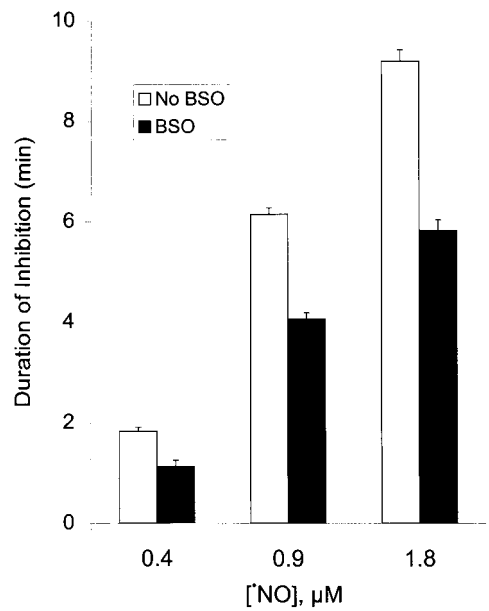


**FIG. 4.** Pretreatment of HL-60 cells with BSO to reduce cellular glutathione decreases the duration of ˙NO inhibition of lipid peroxidation. Cells were incubated in 100 $\mu$M BSO for 24 h prior to determination of the effect of ˙NO on 20 $\mu$M $Fe^{2+}$-induced $O_2$ consumption. Shown are means and standard errors of at least three independent determinations. The differences at 0.4, 0.9, and 1.8 $\mu$M are significant at $P$ = <0.001, <0.001, and 0.004, respectively.

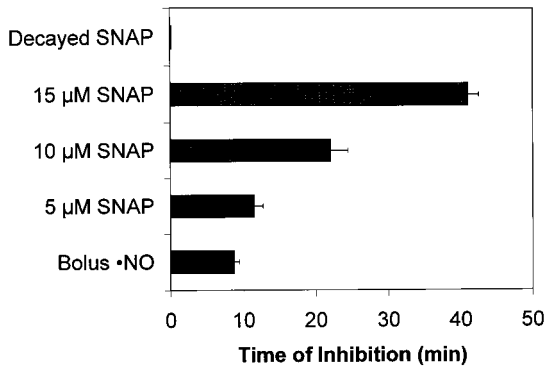


**FIG. 5.** ˙NO donor inhibits lipid peroxidation. Cells were treated as described in the legend to Fig. 1. SNAP was added 1 min after $Fe^{2+}$ and the duration of the inhibition of $O_2$ consumption measured. For comparison, the time of inhibition of bolus ˙NO (1.8 $\mu$M) is also shown. Each data point represents the mean and standard error of at least three independent determinations.

cellular glutathione, and thereby compromising the glutathione peroxidase antioxidant system, diminishes the ability of the cell to cope with an oxidant stress. Thus, ˙NO will be consumed faster due to the increased rate of generation of radicals during cellular lipid peroxidation.

### *Nitric Oxide Generation from ˙NO Donor Compounds also Inhibits Lipid Peroxidation*

In the above experiments, we used an aqueous stock solution of ˙NO to deliver pulses of ˙NO. For comparison, we utilized the ˙NO donor compound SNAP, which achieves more steady and prolonged delivery. SNAP decays to yield ˙NO and *N*-acetyl penicillamine and other potential products, especially in the presence of redox metals such as iron (36). When SNAP was added to cells previously exposed to $Fe^{2+}$ to initiate peroxidation, there was a subsequent inhibition of $O_2$ consumption (Fig. 5). These results are consistent with those using the ˙NO stock solution. The slower release of ˙NO from SNAP resulted in more prolonged inhibition.

### *Nitric Oxide Protects Cells from Peroxidation-Induced Membrane Damage*

Since ˙NO can quench lipid peroxidation in cells, we hypothesized that for this observation to be of significance it should translate into an important biologically observable event, such as protection from lipid peroxidation-induced membrane damage. The addition of 1.8 $\mu$M ˙NO leads to protection against $Fe^{2+}$-induced membrane damage and an increase in cell survival when compared with control experiments without ˙NO (Fig. 6). The protection was statistically significant at both 1.8 and 0.9 $\mu$M ˙NO. These observations are consistent with studies using fibroblasts by Wink *et al.*, who found that ˙NO protects against the toxicity of superoxide and hydrogen peroxide (14). Thus, ˙NO is an efficient antioxidant protecting the integrity of cell membranes that are subjected to an oxidative stress.

### *Significance*

The results presented here clearly demonstrate that ˙NO stops lipid peroxidation processes in cells. In these experiments, a pulse of ferrous iron was used to initiate peroxidation. Nitric oxide appeared not to influence the chemistry of iron in these experiments, but rather it served as a chain-breaking antioxidant in the propagation phase of lipid peroxidation.

Vitamin E (TOH) is an important lipid-soluble antioxidant and has been traditionally thought to be the principal chain-breaking antioxidant in blood and in lipid structures of cells (37). However, the experiments that lead to this conclusion were done in the absence of ˙NO. From the data presented here in conjunction with kinetic information from the literature, it is possible to make a kinetic estimate of the importance of TOH vs ˙NO as a chain-breaking antioxidant in our experiments with cells. A typical TOH level in cells is 1000 to 2000 PUFA/1 TOH (38). If PUFA constitutes about 22% of the lipids in a cell membrane (22, 38), then there are about 5000 to 10,000 total fatty acid chains/1 TOH. As a first approximation, the mole fraction of TOH in lipid regions of membranes will be

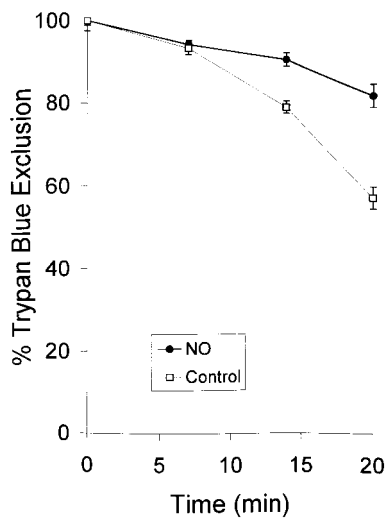


**FIG. 6.** ˙NO protects cells from lipid peroxidation-induced membrane damage. HL-60 cells at $5 \times 10^6$/ml were placed in the oxygen monitor under conditions designed to be similar to those used for $O_2$ consumption experiments in Figs. 1 and 2. They were assayed for trypan blue uptake just before iron addition (time = 0) and then at 7, 14, and 20 min after the initiation of peroxidation. ˙NO (0.9 $\mu$M) was added only once at time = 0. Each data point represents the mean of at least three independent determinations and the bars the standard error. Survival was significantly different at 14 min ($P$ = 0.01) and 20 min ($P$ = 0.005).

$$\text{Mole fraction (TOH)} \approx 1/(5{,}000 \text{ to } 10{,}000)$$
$$\approx 1/7{,}500 \approx 1.3 \times 10^{-4}.$$

Because TOH and the various fatty acids have similar properties, we assume, for simplicity, that they have the same partial molar volume in the lipid regions of cell membranes. Using a density of ≈ 0.9 g/ml and an average molecular weight of 300 g/mole for the fatty acyl chains of the lipids in a cell membrane, the effective molarity of the fatty acyl chains in the lipid regions of membranes will be ≈3 M. The molarity of TOH will then be about

$$3 \text{ M} \times 1.3 \times 10^{-4} \approx 400 \ \mu\text{M}.$$

The rate at which ˙NO would terminate the chain propagation reactions in lipid peroxidation by reacting with the chain-carrying peroxyl radical, LOO˙, reaction [6] above, will be

$$\text{Rate inhibition}_{NO} = k_{NO}[\text{˙NO}][\text{LOO˙}],$$

where $k_{NO} = 2 \times 10^9$ M$^{-1}$ s$^{-1}$ (39) and [˙NO] = 8 × 45 nM = 360 nM. Here 8 is the estimated membrane/water partition coefficient for ˙NO (40, 41); 45 nM is the measured ˙NO concentration in the aqueous phase of our cell suspension during the inhibition phase of lipid peroxidation (Fig. 2). Thus,

$$\text{rate inhibition}_{NO} = 2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1} \times 360 \times 10^{-9} \text{ M}[\text{LOO˙}].$$

The rate at which TOH would terminate these reactions, reaction [7] above, would be

$$\text{rate inhibition}_{TOH} = k_{TOH}[\text{TOH}][\text{LOO˙}],$$

where $k_{TOH} = 8 \times 10^4$ M$^{-1}$ s$^{-1}$ (42) and [TOH] = 400 μM.

Thus,

$$\text{rate inhibition}_{TOH} = 8 \times 10^4 \text{ M}^{-1} \text{ s}^{-1} \times 400 \times 10^{-6} \text{ M}[\text{LOO˙}].$$

The ratio of these rates is

$$\text{rate}_{NO}/\text{rate}_{TOH} = 20/1.$$

In fact, if the concentration and kinetic parameters used to make this estimate were to accurately represent these processes in cells, then an aqueous concentration of ˙NO of only 2 nM would provide antioxidant protection for membrane lipids equal to that of vitamin E. This is a remarkably low concentration of ˙NO and is easily achievable in many cells and tissues.

For the removal of LOO˙ and the products of its reactions, three electrons are needed. The reaction of LOO˙ with TOH results in TO˙. The tocopheroxyl radical can initiate lipid peroxidation, albeit slowly; it is removed by ascorbate or reductive enzyme systems (42–44). Tocopherol provides the first electron for the detoxification of LOO˙; the remaining two electrons come from the glutathione system via glutathione peroxidase:

$$\text{LOOH} + 2\text{GSH} \xrightarrow{GPx \text{ or PhGPx}} \text{LOH} + \text{H}_2\text{O} + \text{GSSG}.$$

The routes for the removal of LOO˙ by ˙NO are as yet unknown. In the reaction with LOO˙ nitric oxide provides the first electron for the detoxification of LOO˙ as the oxidation state of nitrogen changes from +2 to +3 when forming LOONO. It was proposed that the O–O bond in the LOONO formed would be quite weak and thus it would thermally undergo homolytic cleavage to produce LO˙ and ˙$NO_2$ (13). These species are quite oxidizing and would lead to additional oxidations. We saw no evidence of this as the rate of oxygen uptake after the depletion of ˙NO was similar to the rate before the introduction of ˙NO (Fig. 1). If LOONO were to produce these two oxidants, the rate of oxygen uptake after the depletion of ˙NO would be expected to increase due to the oxidations initiated by LO˙ and ˙$NO_2$. An alternative route for LOONO would be simple hydrolysis, producing species that are easily handled by cells:

$$\text{LOONO} + \text{H}_2\text{O} \rightarrow \text{LOH} + \text{H}^+ + \text{NO}_3^-.$$

In this mechanism for the elimination of LOO˙, the three electrons needed to remove LOO˙ and produce the final products all come from the nitrogen as its oxidation state changes from +2 in ˙NO to +5 in $NO_3^-$.

The ferrous iron-induced peroxidation in our system may be due to the presence of preexisting cellular lipid hydroperoxides, as well as the oxidants produced by $Fe^{2+}$ and dioxygen (45). Enrichment of cellular lipids with the polyunsaturated fatty acid docosahexaenoic acid would enhance both of these processes. Typical donor antioxidants, such as vitamin E, would generate additional LOOH during the scavenging of lipid peroxyl radicals providing the seed for additional chain processes upon reaction with $Fe^{2+}$. In contrast, the products of the antioxidant action of ˙NO may not be susceptible to metal-dependent oxidation (46). There-

fore, ·NO may be a better antioxidant than vitamin E since it does not form LOOH that would then be available for initiating new radical chain processes.

The data presented in this work for the first time demonstrate directly that ·NO inhibits membrane–lipid peroxidation in cells. This inhibition was demonstrated by the blunting of oxygen consumption (a reactant) and correlated directly with a reduction in TBARS (a product). This protection against lipid peroxidation, as observed by these two markers, also lead to protection of the physical integrity of cell membranes as detected by trypan blue dye exclusion. The very low concentration of ·NO required to stop lipid peroxidation in cells suggests that ·NO may be an important antioxidant *in vivo*.

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