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Oxygen-Dependent Fragmentation of Cellular DNA by Nitric Oxide

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Although active oxygen species and related metabolites, such as nitric oxide (NO), have been postulated to play important roles in the apoptosis of various cells, a precise mechanism leading to cell death remains to be elucidated. Recently we found that the lifetime of NO depends greatly on the concentration of environmental oxygen and that NO reversibly inhibits mitochondrial respiration and ATP synthesis; the inhibitory effect is stronger at physiologically low oxygen tension than under atmospheric conditions (Arch. Biochem. Biophys. 323, 27–32, 1995). The present work describes the effects of the NO-generating agent, 1-hydroxy-2-oxo-3,3-bis(2-aminoethyl)-1-triazene (NOC 18) and oxygen tension on the respiration, ATP synthesis and apoptosis of HL-60 cells. When respiration was inhibited by NOC 18, cellular ATP levels decreased significantly and DNA fragmentation was elici\ted. Both events were enhanced by decreasing oxygen tension and suppressed by adding NO-trapping agents, such as 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO) and oxyhemoglobin. The fragmentation of cellular DNA was inhibited in a dose dependent manner by herbimycin A, a tyrosine kinase inhibitor. Fragmentation of the DNA of HL-60 cells was also induced either by peroxynitrite, superoxide or hydroxyl radical by some mechanism which was diminished by lowering the oxygen tension. These results indicated that the decrease in cellular ATP and activation of tyrosine kinase might play important roles in NO-induced apoptosis particularly under physiologically low oxygen tensions.

Keywords: Apoptosis, DNA fragmentation, oxygen stress, nitric oxide, HL-60 cells, oxygen tension

Abbreviations: NO, nitric oxide; NOC 18, 1-hydroxy-2-oxo-3,3-bis(2-aminoethyl)-1-triazene; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxyl 3-oxide; HbO_2, oxyhemoglobin; NO_2^-, nitrite; $ONOO^-$, peroxynitrite; $O_2^{\cdot-}$, superoxide; $^{\cdot}OH$, hydroxyl radical; MCLA, 2-methyl-6[p-methoxyphenol]-3,7dihydroimidazo [1,2-a]pyrazine-3-one; NOS, nitric oxide synthase; SIN-1, 3-morpholinosydnonimine; HX, hypoxanthine; XO, xanthine oxidase.

INTRODUCTION

Programmed cell death is an active process which eliminates certain cells selective to maintain a dynamic balance between cell proliferation and cell death and is one of the most important events underlying embryogenesis, metamorphosis and hematopoiesis.[1,2] Apoptosis plays a critical role in

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the process of programmed cell death and usually involves fragmentation of genomic DNA by endonuclease which yields a ladder pattern on agarose gel electrophoresis.[3] Based on the observation that reactive oxygen species induce apoptosis by some antioxidant-inhibitable mechanism,[4–6] critical roles of oxidative stress in the induction of apoptosis have been suggested. Nitric oxide (NO), a short-lived gaseous radical synthesized from L-arginine by NO synthase (NOS),[7–10] has been reported to mediate apoptosis of various cells.[11–16] Activated macrophages generate reactive oxygen species including NO and utilize them as essential mediators in the host defense system.[17] Because of the cytotoxic nature of NO, it kills not only bacteria and other target cells but also NO-generating cells, such as activated macrophages. It is reported that NO-induced apoptosis is an energy-requiring active process.[18] Hence, inhibitors of the mitochondrial respiratory chain and oxidative phosphorylation induce apoptosis of various cells by inhibiting ATP synthesis.[19] The cellular level of ATP is an important determinant for cell death either by apoptosis or necrosis.[20] However, only limited information is available for the role of ATP depletion in the induction of apoptosis. NO reversibly inhibited respiration and ATP synthesis in mitochondria and cells, particularly at low oxygen tensions.[21–23] Peroxynitrite ($ONOO^-$), a metabolite of $NO + O_2^{\cdot-}$ also induces apoptosis of both normal and transformed cells.[24,25] Tyrosine kinase has been postulated to be involved in the mechanism of NO-induced apoptosis.[26–28] Despite extensive studies using various cell types, a molecular mechanism of apoptosis induced by NO and related metabolites remains unclear.

Recent studies in this laboratory[21–23] revealed that, although the lifetime of NO is believed to be extremely short,[29] it is fairly long under physiologically low oxygen tensions (~50 μM). Hence, submicromolar levels of NO reversibly inhibit respiration and ATP synthesis in mitochondria under physiologically low oxygen tensions.[21,22] NO also inhibited the endogenous respiration of Ehrlich ascites tumor cells in an oxygen concentration-dependent manner; the inhibitory effect was enhanced by decreasing oxygen tensions.[23] The aim of the present study is to investigate the mechanism of NO-induced apoptosis in HL-60 cells and its dependency on oxygen tension. The results show that NO inhibited respiration and ATP synthesis and induced DNA fragmentation of HL-60 cells, and suggest that this gaseous radical may play a regulatory role in triggering apoptosis particularly under physiologically low oxygen tensions.

MATERIALS AND METHODS

Materials

NOC 18 and SIN-1 were purchased from Dojindo Laboratory (Kumamoto, Japan). NO gas (99% purity) was obtained from Teisan Co., Ltd. (Tokyo, Japan). NO solution was prepared as described previously.[21] RNase A and proteinase K were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of analytical grade and obtained from Nacalai Tesque, Inc. (Kyoto, Japan).

Cell Line

Human promyelocytic leukemia cell line HL-60 was donated by Dr. Saito, Jichi Medical University. Cells were maintained at concentrations of 0.2 ~ 1 × 10^6/ml in RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd.) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin. They were grown in a humidified incubator at 37°C under a 5% CO_2/95% air atmosphere and used for assays during the exponential phase of growth. Cell counts were routinely performed to maintain the low density of the cell population and assayed for viability by the trypan blue exclusion method.[30]

HL-60 cells (3×10^5 cells) were treated in 1.5 ml of RPMI medium containing 1.0% fetal bovine

serum and various reagents and then incubated either under control (20%) or low (1.0%) oxygen tension in an $N_2/O_2/CO_2$ incubator (BNP-110, Tabai Espec Corp., Tokyo). Oxygen concentration was monitored in the effluent from an incubation chamber with an oxygen probe, and was regulated by adjusting the amount of nitrogen. Before adding reagents, preincubation was normally performed for at least 30 min.

Measurement of Oxygen Consumption

HL-60 cells (2×10^7 cells) were harvested and resuspended in 2 ml of Krebs-Ringer phosphate buffer (KRP, pH 7.4) at 37°C. Oxygen consumption was measured polarographically using a Clark-type oxygen electrode fitted with a water-jacketed closed chamber at 37°C as described previously.[31]

Treatment with NO and Active Oxygen Species

HL-60 cells were treated with NOC 18 which spontaneously released NO (half-life = 78 min at pH 7.4 and 37°C).[32,33] To test the specificity of NO action, SIN-1 and two types of NO trapping agents, carboxy-PTIO and oxyhemoglobin (HbO_2), were used. SIN-1 simultaneously generates NO and $O_2^{\cdot-}$ to form $ONOO^-$. Superoxide was generated by 20 μM hypoxanthine (HX) and 2.0 mU/ml xanthine oxidase (XO). Hydroxyl radical (•OH) was produced by adding 10 μM HX, 1.0 mU/ml XO and 1.0 μM Fe^{2+}. The effect of 30 μM hydrogen peroxide (H_2O_2) on the fragmentation of cellular DNA was also tested.

Measurement of NO as Nitrite

NO production from NOC 18 was determined by measuring nitrite (NO_2^-) in the culture medium by the Griess reaction.[34] Briefly, to a 250 μl sample was added an equal volume of Griess reagent (1% sulfanilamide, 0.1% N-naphthylethylenediamine dihydrochloride in 5% H_3PO_4). After incubation for 5 min at 25°C, nitrite concentration was calculated from the absorbance at 550 nm using $NaNO_2$ as the standard.

Measurement of Superoxide

Superoxide ($O_2^{\cdot-}$) generation in HL-60 cells was measured by the method of Nakano[35] using chemiluminescence of 2-methyl-6-[p-methoxyphenol]-3,7-dihydroimidazo[1,2-α]pyrazine-3-one (MCLA).

Measurement of ATP Concentration

After incubation of HL-60 cells (1×10^6/ml) in the presence or absence of 0.5 mM NOC 18 for 4 hr, cells were boiled at 100°C for 2 min. The boiled mixture was centrifuged at 12,000 rpm for 1 min. ATP in the supernatant fraction was determined by the luciferin-luciferase method using an ATP bioluminescence assay kit CLS II.[36]

Extraction and Electrophoresis of DNA

Cellular DNA was extracted with phenol/chloroform (1:1).[37] Briefly, HL-60 cells (2×10^5 cells/ml) treated with either NOC 18 or various active oxygen species were collected by centrifugation at 12,000 rpm for 1 min and the sedimented cells lysed in 20 μl of a lysis buffer (50 mM Tris-HCl, pH 7.8, 10 mM EDTA, 0.5% sodium-N-lauroylsarcosinate). The lysate was treated with 500 μg/ml RNase A at 50°C for 30 min and then with 500 μg/ml proteinase K at 50°C for 1 hr. DNA was extracted with an equal volume of phenol/chloroform/isoamyl alcohol. Total DNA in the aqueous phase was subjected to agarose gel (2%) electrophoresis in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) containing 0.1 μg/ml ethidium bromide. DNA bands were visualized under ultraviolet illumination and photographed on Polaroid type 667 film (3000 ASA).[38] After subtracting background, the relative amounts of DNA fragments were determined by measuring the image intensity using the public domain NIH Image pro-

gram. pUC 19 plasmid was used as molecular weight markers after digesting by *Hinf* I.

RESULTS AND DISCUSSION

Effect of NO on the Oxygen Consumption of HL-60 Cells at Different Oxygen Tensions

Under standard conditions at 37°C, HL-60 cells consumed oxygen at a rate of 9.8 nmoles/min/10^7 cells. NO reversibly inhibited the endogenous respiration of cells (Fig. 1A). At certain times during incubation, the rate of oxygen uptake returned to the control level. The inhibition occurred more strongly when NO was added at lower oxygen tensions; the time required for the disappearance of the inhibitory effect was 2.8 and 9.2 min at oxygen concentrations of 170 and 40 μM, respectively. Cellular respiration was also inhibited by NOC 18 in an oxygen concentration dependent manner (Fig. 1B). Under the present experimental conditions (0.5 mM NOC 18), 2.5 nmoles/min of NO was generated as judged from the occurrence of NO_2^- + NO_3^- in the medium. In the presence of 0.5 mM NOC 18, cellular respiration was completely inhibited at an O_2 concentration of 60 μM. When the oxygen tension was increased to 200 μM, the inhibitory effect became small and was similar to that of the inhibitor initially added at the same oxygen tension. Thus, the strong effects of NO and NOC 18 at low oxygen tension might not reflect the change in the activity of cell respiration.

Effect of NOC 18 on the ATP Content of HL-60 Cells

To test the effect of NOC 18 on the energy metabolism of HL-60 cells, ATP levels were determined in the presence or absence of 0.5 mM NOC 18. Figure 2 shows the cellular ATP levels after incubation in the presence or absence of NOC 18. At 1 and 4 hr after incubation in 1% oxygen, oxygen concentrations in the culture medium decreased to 90 and 50 μM, respectively. The ATP level in control cells incubated under 20% oxygen was

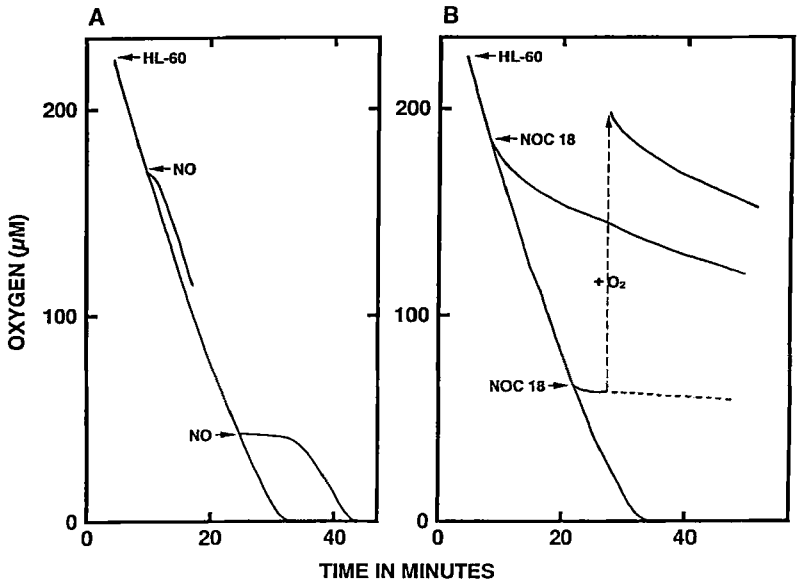

FIGURE 1 Oxygen-dependent inhibition of HL-60 cell respiration by NO and NOC 18. Oxygen consumption in HL-60 cells was measured polarographically using a Clark-type oxygen electrode fitted to a 2-ml water-jacketed closed chamber at 37°C. Harvested cells (1.0×10^7 cells/ml) were resuspended in KRP (pH 7.4). At the indicated time, 0.5 mM NOC 18 or NO (1.9 μM) as NO saturated aqueous solution (1.9 mM) was added. A and B show the effect of NO and NOC 18, respectively.

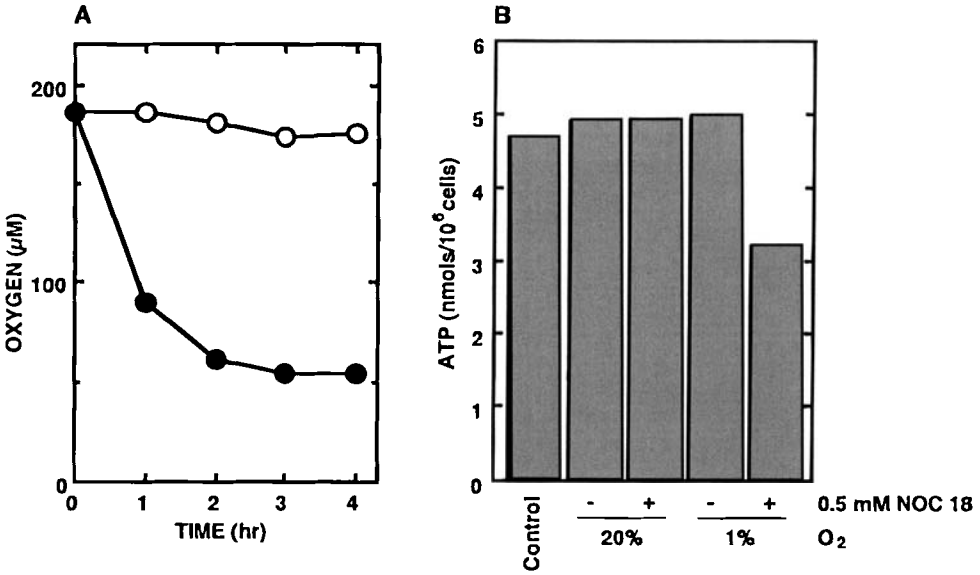

FIGURE 2 Changes in oxygen concentration and ATP content of HL-60 cells after incubation with NOC 18 at high and low oxygen tensions. HL-60 cells (1×10^6 cells/ml) were incubated at high (20%) or low (1%) oxygen tension in the presence or absence of NOC 18 for 4 hr at 37°C. (A) shows the changes in oxygen concentration of the incubation medium in high and low oxygen atmosphere of a $N_2/O_2/CO_2$ culture incubator. Open circles, in 20% oxygen atmosphere; closed circles, in 1% oxygen atmosphere. (B) shows the changes in ATP content in HL-60 cells 4 hr after incubation with or without 0.5 mM NOC 18 in a high or low oxygen atmosphere of a $N_2/O_2/CO_2$ culture incubator.

about 5 nmoles/10^6 cells. The cellular ATP level remained unchanged even after incubation for 4 hr at 1% oxygen. In the presence of 20% oxygen and 0.5 mM NOC 18, no significant change in ATP level was found to occur. However, at 1% oxygen and 0.5 mM NOC 18, the ATP level decreased by about 35%. Although, NOC 18 strongly inhibited the respiration of HL-60 cells at 50 μM oxygen, the cellular ATP level decreased only slightly. Thus, under conditions when cellular respiration was inhibited by NO, the major fraction of ATP in HL-60 cells might predominantly be supplied by glycolysis as observed in Ehrlich ascites tumor cells.[23]

Effect of NO and Oxygen Tension on the DNA Status of HL-60 Cells

To investigate the effect of NOC 18 on cell viability and nuclear events in HL-60 cells at high and low oxygen tensions, the dye exclusion test and agarose gel electrophoresis of DNA were performed. The viability of HL-60 cells and their DNA status were not affected during 4 hr of incubation both under high and low oxygen tensions. However, in the presence of 0.5 mM NOC 18, a marked fragmentation of DNA was induced in an oxygen concentration- and time-dependent manner (Figs. 3 and 4). Furthermore, DNA fragmentation occurred more strongly in culture medium under an atmosphere of 1% oxygen than under 20% oxygen (Figs. 3 and 4, lanes 3 and 8). Thus, NOC 18 induced apoptosis of HL-60 cells depended on the oxygen concentration in the medium. The ability of NOC 18 to induce DNA fragmentation at low oxygen tension but not at high concentration might reflect the oxygen-dependent lifetime of NO derived from its donor.

Effect of Carboxy-PTIO on NOC 18-Induced DNA Fragmentation

To confirm the specificity of NO action for the induction of DNA fragmentation, the effect of car-

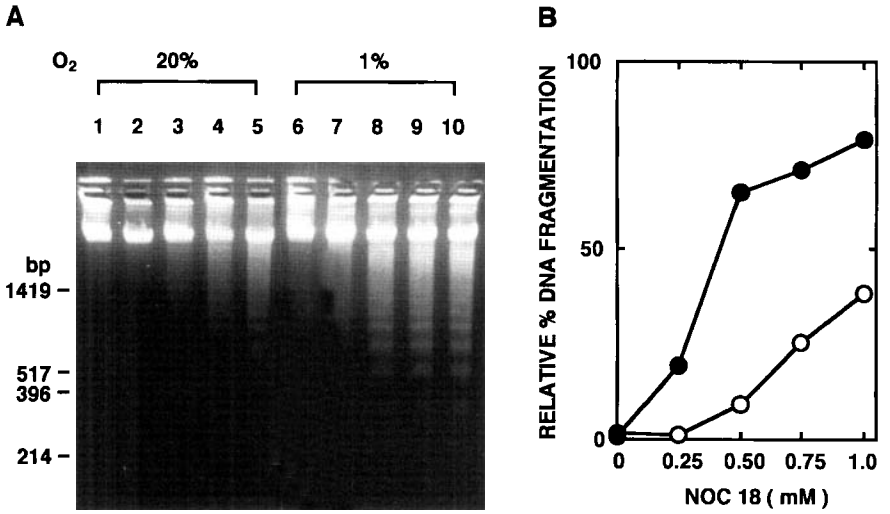

FIGURE 3 Effect of the concentrations of oxygen and NOC 18 on DNA fragmentation. HL-60 cells (2×10^5 cells) were treated with various concentrations of NOC 18 for 4 hr at 37°C under 20% or 1.0% oxygen tensions. The total DNA was then extracted, electrophoresed on 2% agarose gel and visualized by ethidium bromide staining (A). The added NOC 18 concentrations were 0 (lanes 1, 6), 0.25 (lanes 2, 7), 0.50 (lanes 3, 8), 0.75 (lanes 4, 9) and 1.0 mM (lanes 5, 10). The relative % of DNA fragmentation was determined by measuring the image intensity of the lower bands as described in the text (B). Oxygen tensions are 20 (open circles) and 1.0% (closed circles).

boxy-PTIO, a specific compound for trapping NO, was examined. In the presence of 25 ~100 μM carboxy-PTIO, the fragmentation of DNA significantly decreased (Fig. 5). Oxyhemoglobin, another NO trapping agent, also inhibited the fragmentation of DNA almost completely at concentrations higher than 25 μM (data not shown). These results suggested that NO released from NOC 18 might be responsible for the induction of DNA fragmentation in HL-60 cells.

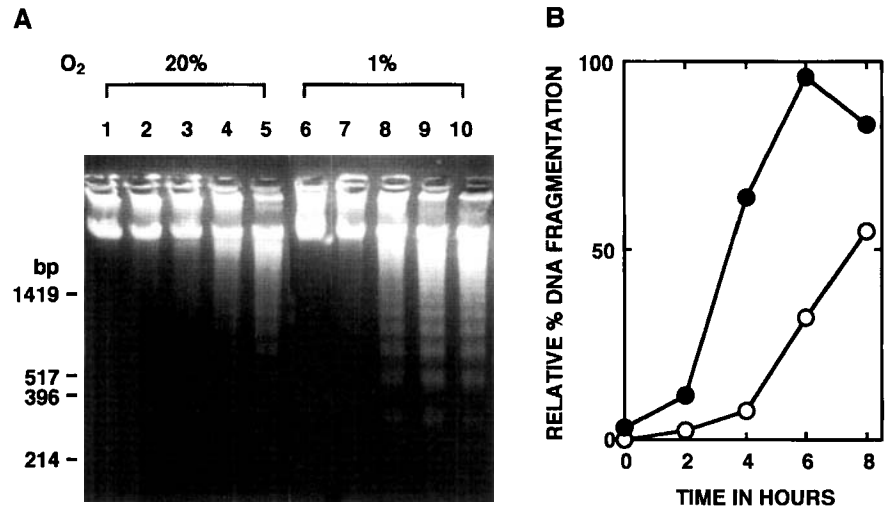

FIGURE 4 Time dependent fragmentation of DNA induced by NOC 18. (A) Agarose gel electrophoresis was carried out with the total DNA extract from cells treated with 0.50 mM NOC 18 for 0 (lanes 1, 6), 2 (lanes 2, 7), 4 (lanes 3, 8), 6 (lanes 4, 9) and 8 hr (lanes 5, 10). Experimental conditions were as described in Figure 2. (B) Relative % of DNA fragmentation. Open and closed circles indicate results obtained from experiments using 20 and 1.0% oxygen tension, respectively.

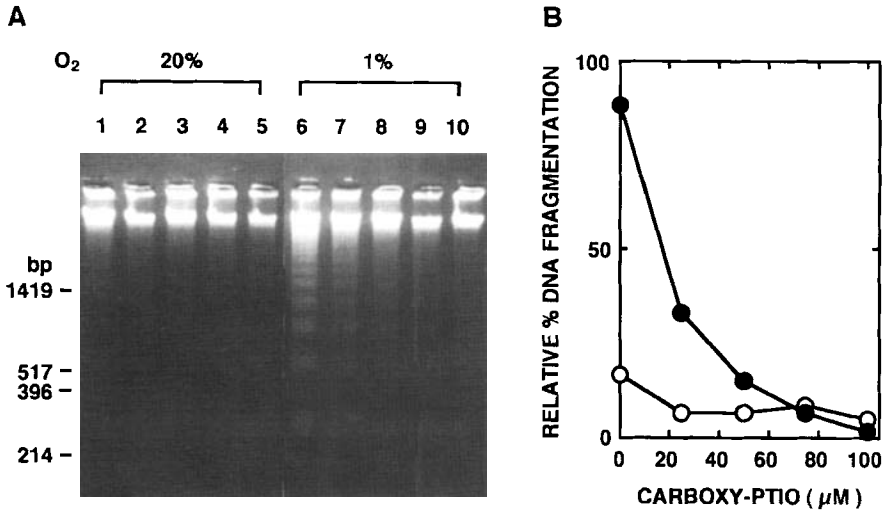

FIGURE 5 Effect of carboxy-PTIO on NOC 18-induced DNA fragmentation. (A) HL-60 cells were treated with 0.50 mM NOC 18 in the presence of carboxy-PTIO. Conditions were the same as described in Figure 2. The added concentrations of carboxy-PTIO were 0 (lanes 1, 6), 25 (lanes 2, 7), 50 (lanes 3, 8), 75 (lanes 4, 9) and 100 μM (lanes 6, 10). (B) Relative % of DNA fragmentation. Open and closed circles indicate the results obtained from experiments using 20 and 1.0% oxygen tension, respectively.

Effects of Nitrite and Peroxynitrite on DNA Fragmentation

NO is metabolized to NO_2^- and $ONOO^-$ by reacting with O_2 and $O_2^{\cdot-}$, respectively. In the absence of NOC 18, no significant amounts of $O_2^{\cdot-}$ and NO were detected by the MCLA-chemiluminescence method and Griess reaction, respectively, presumably due to efficient dismutation of $O_2^{\cdot-}$ by cellular SOD. When the respiration was inhibited by NO, $O_2^{\cdot-}$ generation in mitochondria might be enhanced. Hence, $ONOO^-$ would be formed by the reaction between $O_2^{\cdot-}$ and NO derived from NOC 18. Since $O_2^{\cdot-}$-dependent chemiluminescence of MCLA is quenched by NO, it was practically difficult to measure $O_2^{\cdot-}$ under the present experimental conditions. Thus, to elucidate the possible involvement of NO_2^- and $ONOO^-$, the effects of exogenously added NO_2^- and SIN-1 on the DNA status of HL-60 cells were tested. SIN-1 induced the fragmentation of DNA only slightly (Fig. 6). In contrast, NO_2^- had no appreciable effect on cellular DNA even at high concentrations (~1.0 mM) (data not shown). These results suggested that $ONOO^-$ but not NO_2^- might be involved, at least in part, in the fragmentation of DNA.

Effects of Active Oxygen Species and Oxygen Tension on DNA

Since oxygen stress may underlie the mechanism of apoptosis in HL-60 cells, the ability of NO and other active oxygen species to induce DNA fragmentation were compared (Fig. 6). Superoxide, hydroxyl radical and hydrogen peroxide (H_2O_2) induced fragmentation of cellular DNA as did NO. However, unlike the experiments with NO, DNA fragmentation induced by either $O_2^{\cdot-}$ or $^{\cdot}OH$ was suppressed by lowering the oxygen concentration in the medium. Thus, under physiologically low oxygen concentrations, NO induced apoptosis more effectively than did other species tested. In contrast, DNA fragmentation induced by H_2O_2 was not affected by oxygen tension. Furthermore, DNA fragmentation induced by a fairly high concentration of SIN-1 (0.5 mM) was very weak and affected by oxygen concentration only slightly. Thus, $ONOO^-$ might

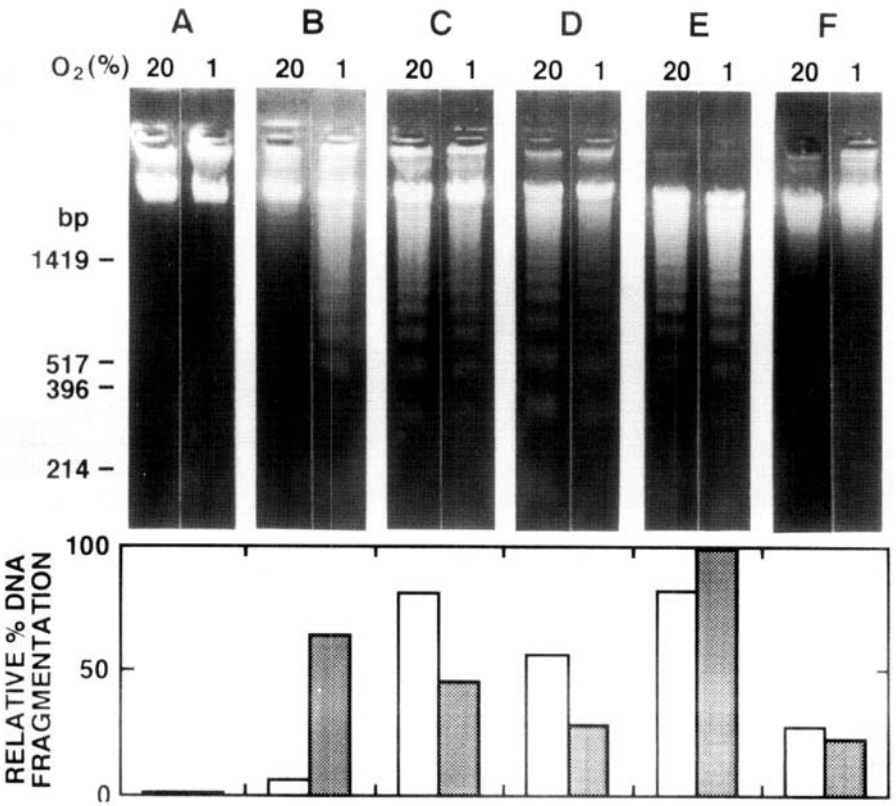

FIGURE 6 Effect of oxygen concentration on DNA fragmentation induced by various agents. Agarose gel electrophoresis was carried out on the total DNA extracted from HL-60 cells (2×10^5 cells) after treating (4 hr) with medium only (A), 0.50 mM NOC 18 (B), 20 μM HX and 2.0 mU/ml XO (C), 10 μM HX, 1.0 U/ml XO and 1.0 μM Fe^{2+} (D), 30 μM H_2O_2 (E) and 0.50 mM SIN-1 (F) under high (20 %) or low (1.0 %) oxygen tension. Bars show changes in the relative % of DNA fragmentation.

not play a major role in the NO-dependent pathway leading to DNA fragmentation of HL-60 cells under low oxygen tension.

Effect of Herbimycin A on NO-Induced DNA Fragmentation

Recent reports suggested that the signal transduction leading to apoptosis might involve tyrosine kinase.[23–25] Figure 7 shows that NO-induced fragmentation of cellular DNA was inhibited by herbimycin A, an inhibitor of tyrosine kinase, in a concentration-dependent manner. Genistein, another inhibitor of tyrosine kinase, also inhibited the fragmentation though its effect was lower than that of herbimycin A (data not shown). These results suggested that tyrosine kinase might also be involved in the mechanism of NO-induced apoptosis of HL-60 cells.

Two possible pathways may underlie the mechanism for NO-induced apoptosis. One possible pathway involves an indirect mechanism, such as glucose starvation and inhibition of mitochondrial respiration,[21–23] and the other involves direct action, such as NO-induced deamination of purine and pyrimidine bases followed by DNA strand breaks.[39] The present experiments revealed an NO- and O_2-dependent fragmentation of HL-60 cell DNA. The oxygen concentration in arterial and venous blood is about 95 (135 μM) and 50 (71 μM) mmHg, respectively. Thus, oxygen tension in and around human leukemic cells might be significantly lower than

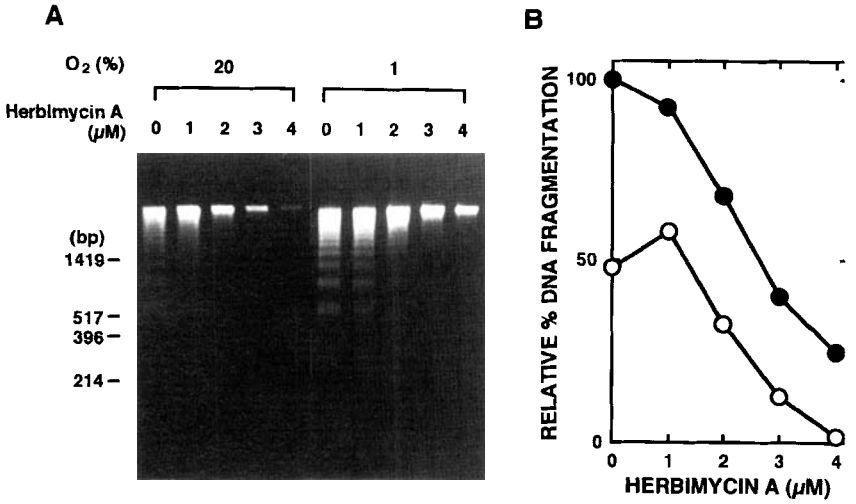

FIGURE 7 Effect of herbimycin A on NO-induced DNA fragmentation of HL-60 cells. Experimental conditions were as described in FIGURE 3. Different concentrations of herbimycin A were added at the same time as 0.5 mM NOC 18 in high and low oxygen atmospheres in a $N_2/O_2/CO$ culture incubator and incubated for 4 hr at 37°C. (A) shows the agarose gel electrophoretic pattern. (B) shows relative % of DNA fragmentation. Open and closed circles indicate the results obtained from experiments using 20 and 1.0% oxygen tension, respectively.

in the atmospheric conditions used for most in vitro experiments. Under physiologically low oxygen concentrations, the respiration and energy transfer reactions of leukemic cells might be inhibited strongly by NO. These results suggested that depression of ATP concentration might underlie the mechanism of NO-induced fragmentation of DNA and apoptotic cell death under physiologically low oxygen tension.

Based on experiments using inhibitors of the synthesis of mRNA and proteins, apoptosis has been suggested to require *de novo* synthesis of some protein(s).[40] Thus, NO might possibly modulate some protein(s) involved in the mechanism for triggering apoptosis, such as G-proteins, protein kinases, phosphatases and transcription factors. Indeed, S-nitrosylation has been shown to inhibit functions of various proteins.[41–44] The present work demonstrates that herbimycin A, an inhibitor of tyrosine kinase, blocked the NOC 18-induced fragmentation of DNA. Thus, as observed with other types of leukocytes,[26–28] tyrosyl phosphorylation of some protein(s) might be involved in the mechanism of apoptosis induced by NO. Recent studies revealed that activation of poly(ADP-ribose)polymerase is involved in NO-induced apoptosis of HL-60 cells.[16] This observation is consistent with the notion described above.

Acknowledgments

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