Free Rad. Res., Vol. 33, pp. 147–156
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Interactions of Nitric Oxide and Oxygen in Cytotoxicity: Proliferation and Antioxidant Enzyme Activities of Endothelial Cells in Culture

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Accepted by Prof. V. Darley-Usmar

(Received 4 November 1999; In revised form 15 December 1999)

Nitric oxide (NO) shows cytotoxicity, and its reaction products with reactive oxygen species, such as peroxynitrite, are potentially more toxic. To examine the role of O_2 in the NO toxicity, we have examined the proliferation of cultured human umbilical vein endothelial cells in the presence or absence of NO donor, ((Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)-amino]diazen-1-ium-1,2-diolate) (DETA-NONOate) (100–500 μM), under normoxia (air), hypoxia (< 0.04% O_2) or hyperoxia (88–94% O_2). It was found that the dose dependency on NONOate was little affected by the ambient O_2 concentration, showing no apparent synergism between the two treatments. We have also examined the effects of exogenous NO under normoxia and hyperoxia on the cellular activities of antioxidant enzymes involved in the H_2O_2 elimination, since many of them are known to be inhibited by NO or peroxynitrite *in vitro*. Under normoxia DETA-NONOate (500 μM) caused 25% decrease in catalase activity and 30% increases in glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities in 24 h. Under hyperoxia NO caused about 25% decreases in activities of catalase, glutathione reductase and glucose-6-phosphate dehydrogenase. The H_2O_2 removal rate by NO-treated cells was computed on the mathematical model for the enzyme system. It was concluded that the cellular antioxidant function is little affected by NO under normoxia but that it is partially impaired when the cells are exposed to NO under hyperoxia.

Keywords: Antioxidant enzymes, endothelial cells, hyperoxia, nitric oxide, oxidative stress, peroxynitrite

INTRODUCTION

Nitric oxide (NO) is a highly diffusible gas which functions as a vasodilator.[1,2] It is produced by many types of cells such as endothelial cells, macrophages and neutrophils. It shows cytotoxicity and inhibits cell proliferation. It has been reported that the cyclic GMP is involved in the growth inhibition of smooth muscle cells.[3,4] NO is capable of reacting with many cellular components containing iron or thiol groups at active sites, which may possibly affect various

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cellular functions. In fact, it has been reported that NO inhibits antioxidant enzymes *in vitro*, such as catalase, glutathione peroxidase (GPx), glutathione-S-transferase and glutathione reductase (GR),[5–9] since they have heme, selenohydryl or sulfhydryl groups at the active sites. GSH is also depleted by NO through the reaction forming S-nitrosoglutathione.[10,11] In addition, it has been shown that sulfhydryl groups are involved in the function of glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD),[12–15] and it is possible that NO inhibits also these NADPH producing enzymes. In view of these results, it seems likely that NO impairs the cellular antioxidant function as a whole and that the malfunction is one of the factors in the NO cytotoxicity. However, the above-mentioned results were all based on the *in vitro* studies using isolated enzymes, and it is important to see whether and to what extent the enzymes are inhibitable in the cellular environments.

When NO is reacted with reactive oxygen species, potentially more toxic species can be formed. According to previous reports, reaction of NO with hydrogen peroxide gives singlet oxygen and that reaction with superoxide (O_2^-) gives peroxynitrite anion ($ONOO^-$).[16,17] Peroxynitrite inhibits isolated GR and GPx.[18–20] It has also been reported that the NO toxicity to cultured cells is potentiated by xanthine oxidase reaction or by hyperoxia,[21–24] and occurrence of peroxynitrite has been assessed in endothelial cells from the generation of nitrotyrosine.[24,25]

In the preceding studies,[26–28] we have examined the dynamics of antioxidant reactions in fibroblasts and endothelial cells in culture. In the vascular system, endothelium is continuously exposed to O_2 and oxidative stress, which may enhance the cytotoxicity of NO. The purpose of this study was to investigate the role of reactive oxygen species in the NO toxicity. It is known that high ambient O_2 concentration accelerates the intracellular generation of reactive oxygen species including superoxide.[29,30] We have compared the effects of NO on the cell proliferation and enzyme activities under normoxic, hyperoxic or hypoxic conditions. Among the enzymes we have focused attention on those participating in the H_2O_2 elimination, because they are crucial to the cellular antioxidant function, and because their activities are most likely affected by NO and its derivatives.

MATERIALS AND METHODS

Chemicals

DETA-NONOate ((Z)-1-[N-(2-aminoethyl)-N-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate) was obtained from Cayman Chemical (Ann Arbor). Diethylenetriamine (bis(2-aminoethyl)-amine), NAD^+ and NADPH were purchased from Wako (Osaka). GSH, GSSG and GSSG reductase were from Sigma (St. Louis).

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics (San Diego) and were cultured as reported previously.[28,31] The culture medium was based on MCDB-107 (Kyokuto Chemical, Tokyo) and contained 15% fetal bovine serum (Filtron, Brooklyn, Australia).

Cell Counting after Culture with NO under Normoxia, Hypoxia or Hyperoxia

HUVEC (population doubling level, 12) were seeded in 35-mm gelatin-coated culture dishes at 2500–3000 cells/cm^2 and were precultured for 1 day. After the culture medium was refreshed, 25 mM DETA-NONOate dissolved in 1 mM NaOH was added to give an appropriate concentration. For experiments under normobaric hyperoxia, the culture dishes were placed in a double-airtight chamber, and humidified O_2 gas containing 5% CO_2 was passed through. The dishes were let stand for 24 h at 37°C with occasional gas flushing. O_2 content of the outflow gas

was monitored with an oxygen analyzer (Model LC-750, Toray Engineering, Ohtsu, Japan) and was found to be between 88% and 94% throughout the treatment. In hypoxic treatment, humidified N_2 containing 5% CO_2 was continuously passed at 100 ml/min over the culture dishes, and O_2 content of the outflow gas was 0.04% or less. In the normoxic experiment, cells were cultured under humidified air containing 5% CO_2 at 37°C.

After the treatments described above, cells in the dishes were washed with phosphate-buffered saline (PBS) and were stained with 0.1% nigrosin. Unstained (viable) cells were counted at ×200 with a microscope (Model TMS-11A, Nikon, Tokyo).

Measurement of Enzyme Activities and Glutathione Content

HUVEC were cultured in 60-mm gelatin-coated dishes to give half to near confluence. After the treatment for 24 h, as described above, cells were scraped off and were suspended in the preparation medium (0.1 M phosphate or Tris–HCl buffer at pH 7.4 containing 0.5% Triton X-100 and 0.1 mM EDTA). After sonication on ice, cell debris was removed by centrifugation at 10,000× g for 15 min at 0°C.

Enzyme assays were carried out at pH 7.4 and 37°C, as described previously.[28] The units of enzyme activities for GPx, GR, G6PD and 6PGD were taken as μmoles of NADPH consumed or formed per minute. The unit for catalase activity was taken as the first-order rate constant (min^{-1}) for H_2O_2 decay. Intracellular glutathione was extracted as reported previously[31] and was determined by the method of Tietze.[32]

Computation of H_2O_2 Removal Rate

The steady-state H_2O_2 removal rate by HUVEC was computed according to the mathematical model, as reported previously.[28] The model included reactions catalyzed by hexokinase, G6PD, 6-phosphogluconolacotonase, 6PGD, GR, GPx and catalase. Rates of the enzyme reactions were calculated on the basis of known steady-state rate equations and parameter values. The maximum velocities for G6PD, 6PGD, GR, GPx and catalase, as well as total glutathione concentration, were taken from the results of this study. Initial concentrations of metabolites and coenzymes, other than that of glutathione, were the same as those used previously, and 75.5% latency in catalase activity was assumed.[28]

Statistical Analyses

Data are expressed as means ± SD, unless otherwise noted. The statistical significance was judged by two-tailed unpaired t-test, and in the figures statistical significance is shown by asterisks (* for $P < 0.05$ and ** for $P < 0.01$).

RESULTS

Effects of NO on the Growth of HUVEC

As an NO donor, we have chosen DETA-NONOate, since it decays slowly enough under physiological conditions,[33] so that it generates NO continuously during the culture (for 24 h). Prior to the experiments, we have measured the decay rate of the NO donor by following the absorbance decrease at 252 nm. Half-lives of the compound at 37°C were found to be 21.5 h in PBS and 12.5 h in the culture medium used in this study (data not shown). The half-life in the culture medium corresponds to a first-order rate constant of $5.56 \times 10^{-2}\,h^{-1}$, and the NO producing rate of 500 μM DETA-NONOate was calculated to be 15.4 nM/s at the start and 4.1 nM/s after 24 h. NO further reacts with O_2 and possibly with other cellular and medium components, and a pseudo-steady-state NO level should be reached. It has been reported that when 500 μM DETA-NONOate was added to a culture medium the NO concentration reaches a maximum (about

1.7 μM) after 1–2.5 h and gradually decreases afterward.[23]

Figure 1 shows the number of viable cells after 24 h of culture in the presence of different concentration of DETA-NONOate (0–500 μM). In this work, we take the results obtained under air as those for "normoxia", since HUVEC is always exposed to relatively high O_2 concentration *in vivo*. In the control experiments (without NO) under normoxia and hypoxia, the final cell density was less than 20% of that at confluence, and no cell death was observed at the cell counting. As seen in Figure 1A, under air the cell number showed about two-fold increase in 24 h, and 100 μM NONOate suppressed the growth to less than half. The cell showed little

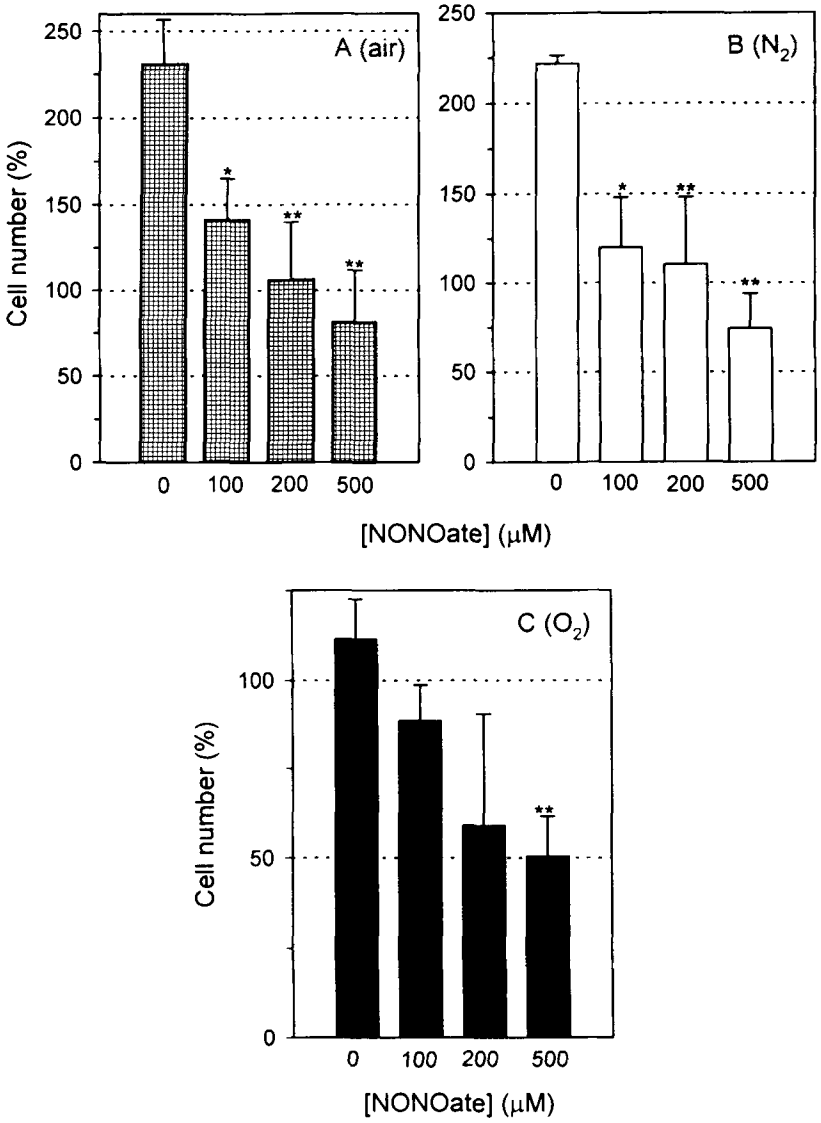

FIGURE 1 Effects of exogenous NO and O_2 on the growth of HUVEC. Cells were cultured for 24 h in the presence of different concentrations of DETA-NONOate. (A) Air + 5% CO_2, (B) 95% N_2 + 5% CO_2, (C) 95% O_2 + 5% CO_2. Results were obtained from triplicate experiments. *Asterisks* show the statistical significance (* for $P < 0.05$ and ** for $P < 0.01$).

growth with 200 μM NONOate, and at 500 μM the cell number decreased slightly. Under a stream of N_2 (Figure 1B), the cell growth was retarded slightly (about 10%) as compared with the growth in air. When cells were cultured under 95% O_2 (Figure 1C), the growth was considerably retarded, and NONOate caused cell death depending on its concentration. Although considerable differences were observed in the cell proliferation, the observed NO dose dependencies were apparently similar under normoxia, hypoxia or hyperoxia.

One mole of DETA-NONOate produces 2 mol of NO and 1 mol of diethylenetriamine; NO is furthermore converted into NO_2^- by reaction with O_2. As a control experiment, the cells were treated with 500 μM diethylenetriamine and 1 mM $NaNO_2$ for 24 h, but no difference in the growth was observed (data not shown).

Effect of NO on the Antioxidant Enzyme Activities of HUVEC

Figure 2 shows the activities of enzymes (catalase, GPx, GR and 6PGD) in HUVEC that were untreated or treated with NONOate under normoxia or hyperoxia. Since the enzyme assays were done in the absence of NONOate, only irreversible changes were detected, such as enzyme induction or inactivation. Although statistically not significant, all the enzyme activities increased slightly under hyperoxia, which is probably due to enhancement of enzyme synthesis, since generally hyperoxia causes induction of the antioxidant enzymes. Catalase activity was significantly diminished by NO (about 25%) under both normoxia and hyperoxia (Figure 2A). Interestingly, GR activity showed a 25% decrease only by combination of NO and hyperoxia, and no such decrease was observed under normoxia (Figure 2C). Both G6PD and 6PGD activities showed approximately 30% increase with NO (Figure 2D and E), but under hyperoxia G6PD activity decreased by 30%, while 6PGD activity remained unchanged with NO under hyperoxia.

Figure 2F shows the glutathione contents of HUVEC. The results accord to those previously reported, which have shown that chronic treatment with NO or hyperoxia increases the glutathione content of cultured cells through the induction of cystine uptake and glutathione synthesis.[34–38] No synergism was observed between NO and hyperoxia in the effect.

DISCUSSION

Effect of O_2 on the NO Toxicity

Narula *et al.*[23] have reported that the effects of NO and O_2 were synergistic on the growth of alveolar epithelial cells in culture, since extensive cell death occurred in the presence of an NO donor under hyperoxia. It is rather difficult, however, to analyze the effects quantitatively, since the cell growth and death take place simultaneously in those experiments. In our study, decrease in cell number was also observed, particularly in the presence of both NO and high O_2. Nevertheless, the NO dose dependencies were similar at different concentrations of ambient O_2 (Figure 1), and we conclude that no apparent synergism was observed between NO and O_2, as far as the proliferation of HUVEC is concerned. The interactions between NO and oxygen species may still exist in cellular environment, but in any case, the effects were not detectable in terms of cell proliferation.

Effects of NO on Antioxidant Enzyme Activities

After the culture of HUVEC with NO, catalase activity showed a considerable decrease (Figure 2). The present results are concerned only with irreversible changes, since the enzyme assay was performed in the absence of NO. It has been reported that NO reversibly binds to catalase and inhibits it with inhibition constant of 0.18 μM.[5] When catalase activity of cell homogenate was measured in the presence of

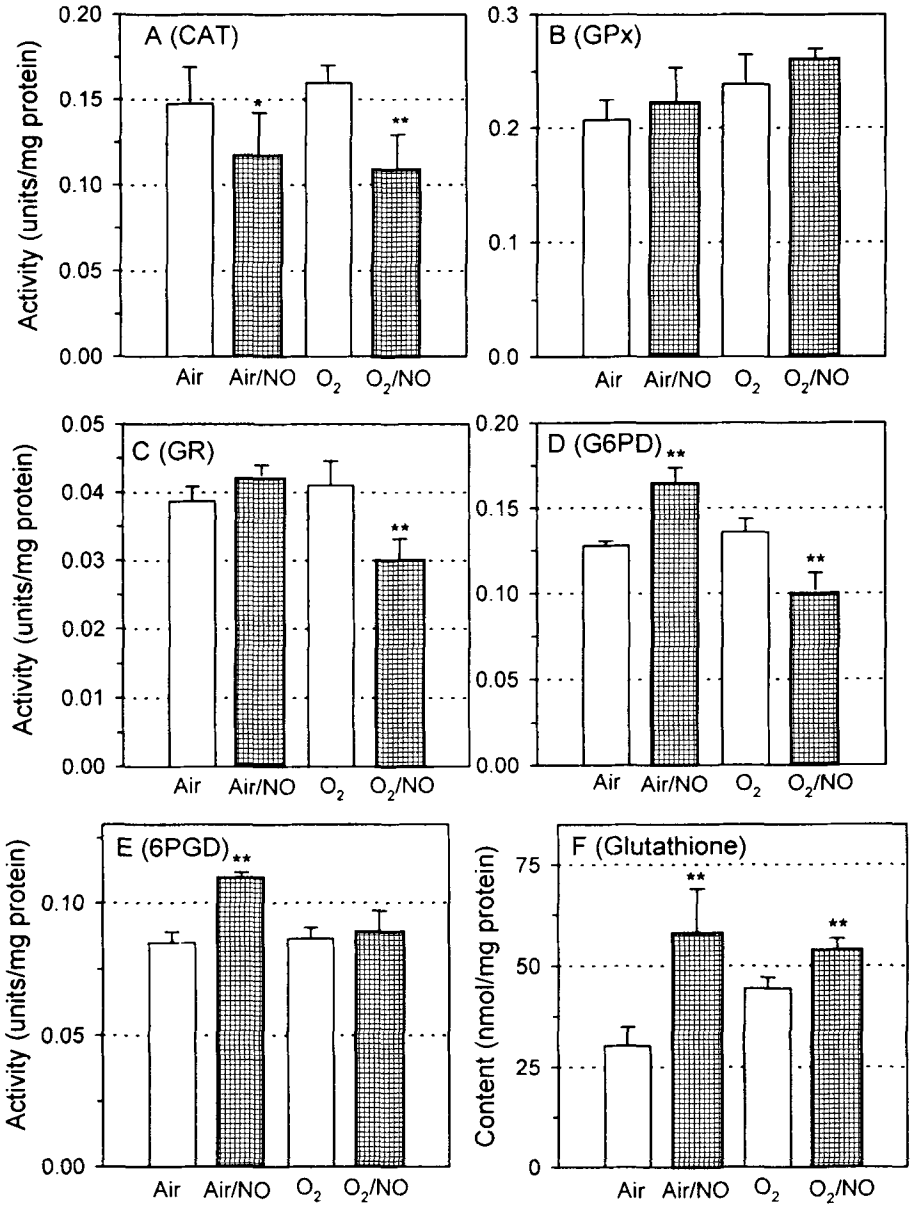

FIGURE 2 Effects of NO and hyperoxia on the enzyme activities and glutathione content of HUVEC. "CAT", "Air", "O_2" and "NO" stand for catalase, normoxia, hyperoxia and DETA-NONOate treatment respectively. Initial NONOate concentration was 500 μM. Results were obtained from quadruplicate experiments. *Asterisks* show the statistical significance of the differences between NO-treated and untreated cells under the same O_2 concentration.

500 μM NONOate, the activity was inhibited by only 20% at most (data not shown). The degree of inhibition was considerably lower than that expected from the steady-state NO concentration (about 1.7 μM),[23] and the results indicate that NO concentration is lower than expected in cellular environment or that catalase is less inhibitable there. In any case, the present results indicate that some irreversible changes had occurred in catalase, probably through modification of the

heme moiety or decrease in the transcriptional regulation. Since the degrees of activity changes were similar in normoxic and hyperoxic environments, it is unlikely that O_2 or its derivatives are involved in the process.

GPx activity showed a slight increase when cells were exposed to hyperoxia or NO for 24 h (Figure 2B), probably because of enzyme induction. Asahi *et al.*[6,39] have reported that an NO donor rapidly inactivates the purified enzyme *in vitro* through oxidation of cysteine or selenocysteine. In this study, no such inactivation was observed under both normoxia and hyperoxia. The discrepancy may be ascribed to difference in GPx reactivity between cellular and *in vitro* environments or to a side effect of the S-nitrosothiol compound *in vitro*.

GR activity showed a significant decrease when cells were exposed to NO under hyperoxia, but no such inactivation was observed under normoxia (Figure 2C). The properties also differ from those of isolated GR, which is irreversibly inactivated by NO under normoxic conditions.[7–9] It is suggested that O_2 or its derivatives are involved in the inactivation of GR in the cell.

G6PD activity also showed a significant reduction in the presence of NO under hyperoxia. This is probably due to modification of the sulfhydryl groups, which are implicated in the enzyme function.[12,13]

Effects of NO on the H_2O_2 Removing Activity

In living cells H_2O_2 is eliminated mainly by catalase at high H_2O_2 concentrations ($> 100\,\mu M$) and mainly by GPx at low concentrations ($< 100\,\mu M$).[26,28] Our previous studies[27,28] have shown that GR reaction is the primary rate-limiting step and that G6PD is secondary in the GSH-dependent removal of peroxides. In the present study, under air catalase activity was decreased by NO, but G6PD and 6PGD activities were considerably increased (Figure 2). Since changes in the other enzyme activities were small, it is estimated that the H_2O_2 removing activity is not much affected as a whole. On the other hand, when cells are exposed to NO under hyperoxia, it is thought that H_2O_2 removing activity is diminished as a result of loss in activities of the three key enzymes, catalase, GR and G6PD. Possibly, other GSH-dependent reactions are also depressed because of decline in the glutathione recycling activities.

In the previous study,[28] we constructed a mathematical model describing the steady-state H_2O_2 elimination by HUVEC. The model calculation seems useful for the present study, since several changes take place simultaneously in the enzymes and coenzyme participating in the metabolism. To apply the model to the present case, we set the maximum velocities of the enzymes and glutathione concentration equal to those found in this study (Figure 2), while other parameter values were set equal to those employed previously.[28] The steady-state H_2O_2 removal rates were obtained for different H_2O_2 concentrations (1, 10 and 100 μM), and the results are shown in Figure 3. It is seen that at $[H_2O_2] \leq 10\,\mu M$ GPx reaction predominates over catalase reaction. At 1 μM H_2O_2 the GSH-dependent H_2O_2 removal rate parallels GPx activity (cf. Figure 2B), since glutathione recycling is not rate limiting. At 100 μM H_2O_2 the GSH-dependent rate parallels GR activity (cf. Figure 2C), since the GR reaction becomes rate limiting. Under normoxia, NO little affected the total rate of H_2O_2 removal. Under hyperoxia, the removal rate at 100 μM H_2O_2 is evidently diminished by NO, and it was confirmed that the antioxidant activity is considerably diminished under the conditions. Glutathione content itself does not appreciably affect the rate, since its concentration is well over the K_m value for GR.

NO acts as antioxidant as well as pro-oxidant, depending on the situation. It exerts protective effects against H_2O_2 toxicity, when it is generated at controlled rate for sufficiently long period.[40] From the results of the present study,

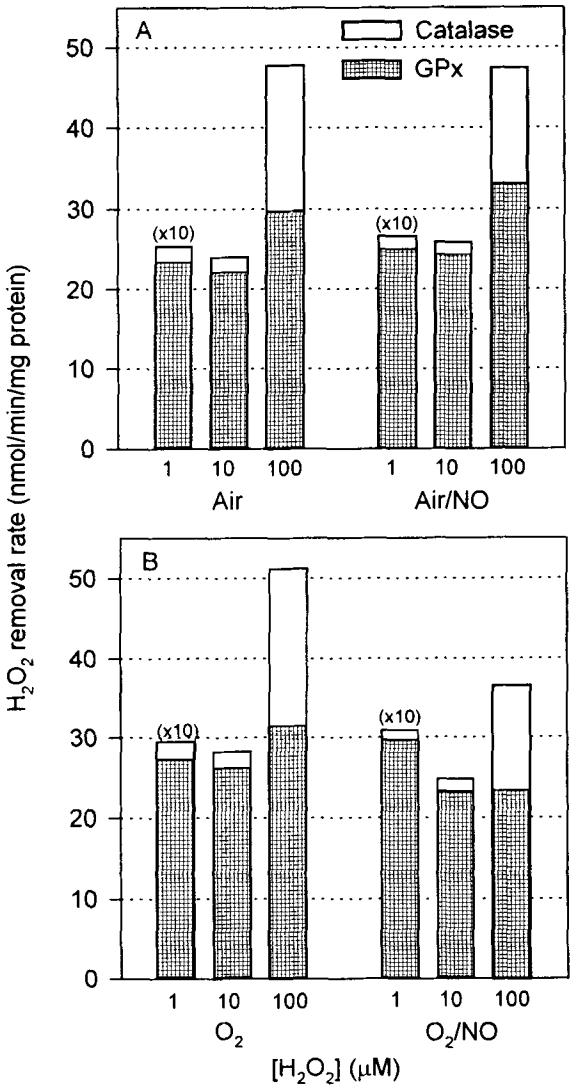

FIGURE 3 Steady-state H_2O_2 removal rates computed according to the mathematical model[28] for different H_2O_2 concentrations (1, 10 and 100 μM). Total glutathione content and maximum activities of catalase, GPx, GR, G6PD and 6PGD were set equal to those found in this study. For catalase activity 75.5% latency was assumed.[28] *Hatched* and *open bars* show the H_2O_2 removal rates by GPx and catalase respectively. "Air", "O_2", and "NO" stand for normoxia, hyperoxia, and treatment with 500 μM DETA-NONOate respectively. For 1 μM H_2O_2 the ordinate was multiplied by the factor of 10.

it is inferred that NO does not inhibit the antioxidant enzymes under ordinary conditions, and on the contrary it is expected that NO adds to antioxidant capacity of the cell.

CONCLUSION

Synergism between NO and O_2 was not detectable in terms of the endothelial cell proliferation. The effects of NO on the antioxidant enzymes in cellular environment are considerably different from those observed *in vitro* experiments using isolated enzymes. NO itself does not impair the cellular antioxidant capacity significantly, but its interactions with oxygen, or more possibly interactions with reactive oxygen, lead to the impairment of the function.

Acknowledgments

This work was supported partly by the Ibaraki Prefectural University Project Research.

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