Free Rad. Res., Vol. 34, pp. 193-202
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The Role of Nitric Oxide in Paraquat-Induced Cytotoxicity in the Human A549 Lung Carcinoma Cell Line

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Accepted for publication by Prof. E. Niki

(Received 14 April 2000; In final form 22 May 2000)

Paraquat (PQ) is a well-known pneumotoxicant that exerts its toxic effect by elevating intracellular levels of superoxide. In addition, production of pro-inflammatory cytokines has possibly been linked to PQ-induced inflammatory processes through reactive oxygen species (ROSs) and nitric oxide (NO). However, the role of NO in PQ-induced cell injury has been controversial. To explore this problem, we examined the effect of NO on A549 cells by exposing them to the exogenous NO donor NOC18 or to cytokines; tumor necrosis factor-α, interleukin-1 β and interferon-γ, as well as PQ. Although the exogenous NO donor on its own had no effect on the release of lactate dehydrogenase (LDH), remarkable release was observed when the cells were exposed to high concentrations of NOC18 and PQ. This cellular damage caused by 1 mM NOC18 plus 0.2 mM PQ was ascertained by phase contrast microscopy. On the other hand, NO derived from 25–50 μM NOC18 added into the medium improved the MTT reduction activity of mitochondria, suggesting a beneficial effect of NO on the cells. Incubation of A549 cells with cytokines increased in inducible NO synthase (iNOS) expression and nitrite accumulation, resulting in LDH release. PQ further potentiated this release. The increase in nitrite levels could be completely prevented by NOS inhibitors, while the leakage of LDH was not attenuated by the inhibition of NO production with them. On the other hand, ROS scavenging enzymes, superoxide dismutase and catalase, inhibited the leakage of LDH, whereas they had no effect on the increase in the nitrite level. These results indicate that superoxide, not NO, played a key role in the cellular damage caused by PQ/cytokines. Our in vitro models demonstrate that NO has both beneficial and deleterious actions, depending on the concentrations produced and model system used.

Keywords: A549 cells; paraquat poisoning; nitric oxide; cytokine; free radical

INTRODUCTION

Paraquat (PQ; methyl viologen : 1,1′-dimetyl-4,4′-bipyridylium ion) is a widely used herbicide that causes lethal intoxications in mammals. The lung is the most severely affected organ, and lung involvement is responsible for the high mortality from PQ poisoning. Since PQ easily undergoes redox cycling, it has been proposed that reactive oxygen species (ROSs), such as superoxide, hydrogen peroxide and hydroxyl radical, cause injury to the target organ after PQ intake.[1,2] However, the precise biochemical

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events are still controversial. Besides a lipid peroxidation mechanism, available evidence indicates that other mechanisms may be also involved.[3,4] Berisha et al.[5] reported that nitric oxide (NO) played a critical role in the oxidant injury induced by PQ. Other investigators[6] reported that the effect of PQ depended on obstruction of electron transfer by respiratory chain inhibition by NO. There is now increasing evidence to suggest that NO and its derivatives, such as peroxynitrite, play an important role in cytotoxicity, especially under a condition in which superoxide and NO exist together.[7,8] On the other hand, NO has been reported to be protective against oxidative stress.[9–11] NO inhalation has been applied to the treatment of severe PQ poisoning.[12]

In this study, we examined the involvement of NO on PQ-induced cell injury using a human lung carcinoma A549 cell line. As alveolar type II cells are a target for pneumotoxicants such as PQ[13] and they are believed to be derived from human alveolar type II cells[14], we used this cell line as our cellular model. Using an NO donor, NOC18, or cytokine mixture in the presence or absence of PQ, we assessed the release of lactate dehydrogenase (LDH) as a marker for membrane damage and the reduction of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) for mitochondrial activity.

MATERIALS AND METHODS

Materials

Paraquat (PQ; methyl viologen), superoxide dismutase (SOD; from bovine erythrocytes), catalase (from bovine liver), and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St Louis, MO). The nitric donor, NOC18, was obtained from Dojindo Laboratories (Kumamoto, Japan). N^G-monomethyl-L-arginine acetate (L-NMMA) was obtained from Research Biochemicals Incorp (Natick, MA). Recombinant human tumor necrosis factor-α (TNF-α), interleukin –1ß (IL-1ß) and interferon-γ (IFN-γ) were obtained from Genzyme (Cambridge, MA). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from ICN Biomedicals Japan (Tokyo, Japan). [α-^{32}P] dCTP(specific activity 10mCi/ml) was obtained from the Radiochemical Center, Amersham Japan (Tokyo, Japan). A probe for the human inducible nitric oxide synthase (iNOS) gene was kindly provided by Dr. T. Ogura (National Cancer Center Research Institute, East, Chiba, Japan) and Dr. S.C. Erzurum (Cleveland Clinic Foundation, Cleveland, OH). All other reagents employed were of analytical reagent grade.

Cell culture

A549 cells, a human alveolar type II epithelium-like cell line derived from a lung adenocarcinoma, were obtained from the RIKEN Cell Bank and cultured in DMEM medium supplemented with 0.2 mM L-glutamine, and 10% heat-inactivated FBS at 37 °C in a humidified atmosphere of 5% CO_2 in air. They were routinely subcultured twice a week by trypsinization and seeding.

Cell stimulation and biochemical assays

Cells (3×10^5/well) were plated in 12-well plates containing DMEM medium supplemented with 10% FBS and allowed to adhere. After 18 hr, the medium was replaced with phenol red-free DMEM containing 5% FBS and 1 mM L-arginine, and the cells were exposed to additives for 24 hrs. The stock solution of NOC18 was prepared in 0.1 M NaOH and was diluted with buffered saline immediately before use. Since incubation with the cytokine mixture resulted in a larger increase in nitrite accumulation than with each

cytokine alone, we added the cytokine mixture to the medium as follows; IL-1ß; 10 units/ml, TNF-α; 10 ng/ml, IFN-γ; 500 units/ml. Nitrite in the medium was determined by the Griess reaction (NO_2/NO_3 Assay Kit-C; Dojindo Laboratories, Kumamoto, Japan) and lactate dehydrogenase (LDH) activity in the medium was measured spectrophotometrically using a Test Kit (Kyokuto Seiyaku Co., Tokyo, Japan).

Mitochondrial metabolic activity was estimated by the reduction of MTT to its corresponding blue formazan product according to a procedure described elsewhere.[15] Cells ($1x 10^4$/well) were plated into each well of a 96 well-plate in 0.1 ml DMEM containing 5% FBS. After 24 hr incubation, PQ and NOC18 at various concentrations were added successively to each well. At 18 hr after exposure to additives, the cells were further incubated for 4 hr with 0.5 mg/ml MTT. At the end of the incubation period, the cells were washed twice with normal saline and lysed in 0.1 ml of acidified (0.04 M HCl) isopropyl alcohol. Absorbances were read within an hour on an ELISA plate reader (BIO-RAD Model450) with a test wavelength of 570 nm and a reference wavelength of 630 nm.

Northern blot analysis

Twenty μg equivalents of total cellular RNA were denatured in formaldehyde/ formamide by heating at 65 °C for 15 min, separated on denaturing 1% agarose gels and transferred to Hybond N+ membranes (Amersham). After baking followed by prehybridization, hybridization was carried out in a solution containing ^{32}P-labeled iNOS cDNA at 42 °C overnight. After hybridization, filters were washed and exposed to a Fuji imaging plate (BAS-III), and the radioactivities of the plates were measured by an imaging analyzer (BAS 2000, Fuji Photo Film Co. Ltd., Tokyo, Japan). To ensure equal transfer of the RNA to the membrane, the membrane was stripped by boiling in 0.1% SDS and reprobed with a probe for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA (Clontech).

RESULTS

Effect of the exogenous NO on membrane damage

A549 cells were treated with increasing concentrations of NOC18 with or without 0.2 mM PQ. The nitrite level in the medium dose-dependently increased with the addition of NOC18 and was independent of PQ (Fig. 1). There was no damage to the permeability of the plasma membrane with NOC18 alone, but when the cells were exposed to PQ and the NO donor above a 1 mM concentration, there was remarkable damage (Fig. 2). The cells were disrupted and showed regressive changes, and nuclei became pycnotic by incubation with NOC18 (1 mM) plus PQ (0.2 mM). In contrast, the cells incubated with NOC18 (1 mM) or PQ (0.2 mM) alone showed normal morphology (Fig. 3). These observations were mimicked by another NO donor, sodium nitroprusside.

Effect of the exogenous NO on mitochondrial activity

Mitochondrial dehydrogenase activity, the cleavage of MTT into insoluble formazan, dose-dependently decreased in the presence of PQ from 0.1 to 0.5 mM. On the other hand, a slight increase in MTT reduction activity was observed in each PQ group when 25 – 50 μM NOC18 was added to the medium, indicating that a small level of NO had a beneficial effect (Fig. 4). Above these concentrations of NOC18, the mitochondrial activity decreased in the dose-dependent manner of the NO donor.

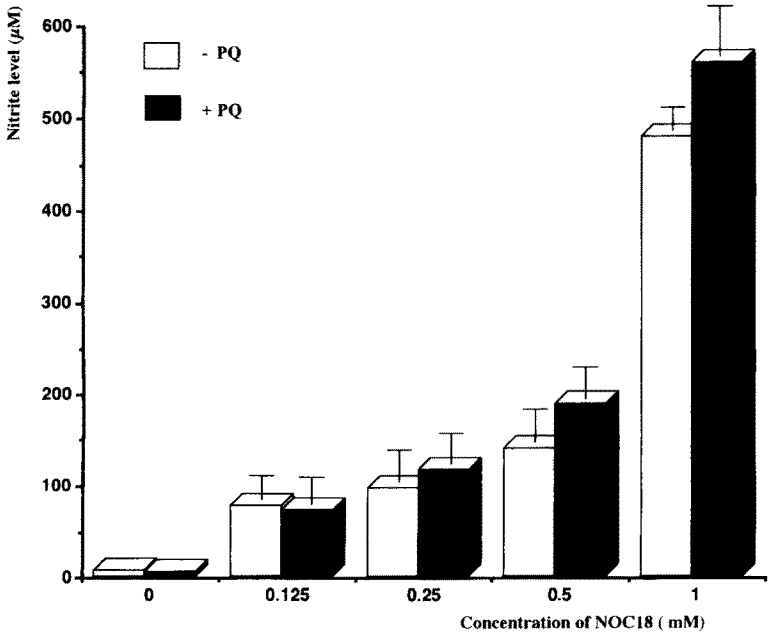

FIGURE 1 Nitrite accumulation in the medium caused by the addition of NOC18 in the presence or absence of 0.2 mM PQ. A549 cells plated in 12-well plates (3×10^5/well) were incubated with the additives for 24 hrs. Data are represented as mean ± S.D. (n= 4)

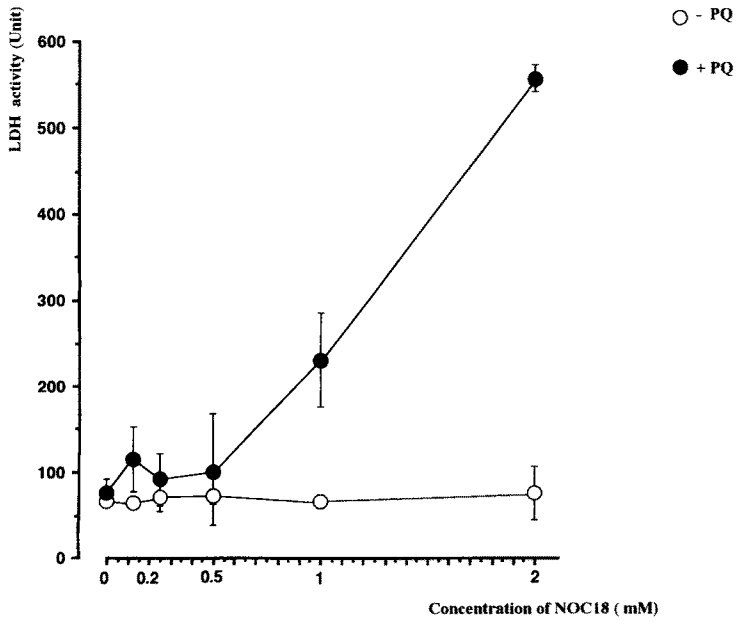

FIGURE 2 Effect of NOC18 on the release of lactate dehydrogenase (LDH) into the medium in the presence or absence of 0.2 mM PQ. A549 cells plated in 12-well plates (3×10^5/well) were incubated with the additives for 24 hrs. Data are represented as mean ± S.D. (n= 4)

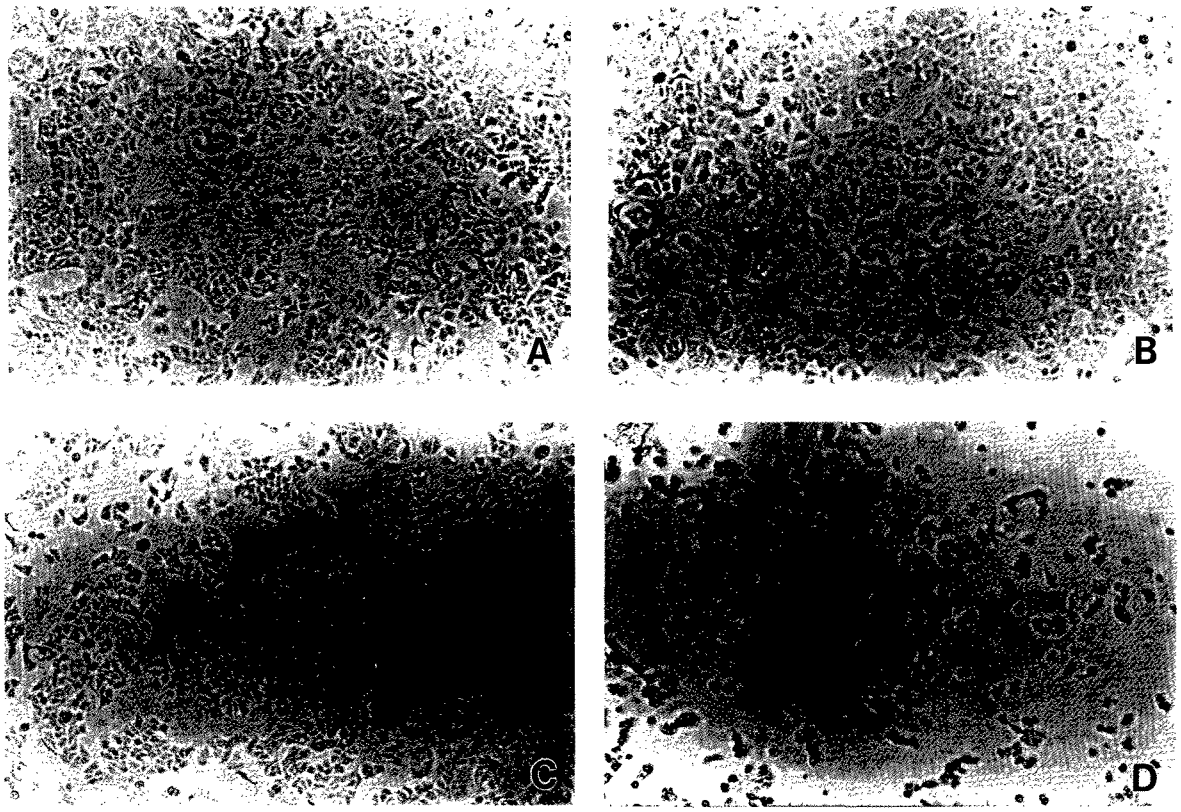

FIGURE 3 Photomicrographs showing the effect of 1 mM NOC18 on cell morphology in the presence or absence of 0.2 mM PQ. (A) control, (B) 1 mM NOC18, (C) 0.2 mM PQ, (D) 1 mM NOC18 / 0.2 mM PQ. Although the cells treated with NOC18 or PQ alone remained normal, those treated with NOC18 / PQ showed a dramatic damage

Effect of endogenous NO on membrane damage

We added cytokines to A549 cells to induce inducible NO synthesis (iNOS). The level of iNOS mRNA of the cells treated with cytokines and/or PQ (0.2 mM) is shown in Fig. 5. Although no expression was observed with PQ alone, it was not inhibited by PQ. Control experiments demonstrated equivalent expression of the GAPDH gene in all samples. The iNOS mRNA became detectable within 3 hrs, and reached a maximum level within 6–9 hrs. The nitrite level was 3.7 ± 0.4 μM in the medium with no additives and it significantly increased with exposure to cytokines (10.9 ± 1.1 μM) or PQ/cytokines (12.3 ± 1.8 μM) (Fig. 6). The level was depressed by a NOS inhibitor, L-NMMA (1 mM), whereas ROS scavenging enzymes, SOD /catalase (200 units/160 units), showed no effect. We examined the effects of SOD/catalase and NOS inhibitors on the LDH release caused by PQ/cytokines. Fig. 7 shows the LDH activity in the medium after incubation with additives. A slight increase in LDH release was observed with 0.2 mM PQ alone. Cytokines potentiated the release and PQ/cytokines further potentiated it. The NOS inhibitor L-NMMA had no effect on the LDH release into the medium caused by PQ/cytokines. Other NOS inhibitors, such as N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-nitro L-arginine (L-NNA), also had no effect (data not shown). ROS scavenging enzymes, in contrast, significantly sup-

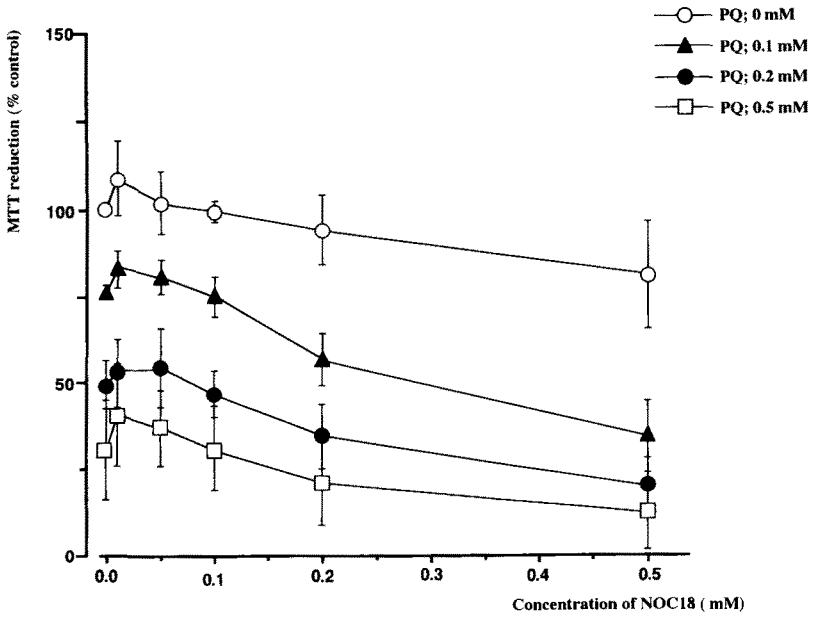

FIGURE 4 The MTT assay. A549 cells plated in 96-well plates (1×10^4/well) were incubated with the additives for 18 hrs. The cells were further incubated for 4 hrs with 0.5 mg/ml MTT. Results are expressed in percentage of the amounts of formazan produced by control cultures (defined as 100%). Data are represented as mean ± S.D. (n= 6)

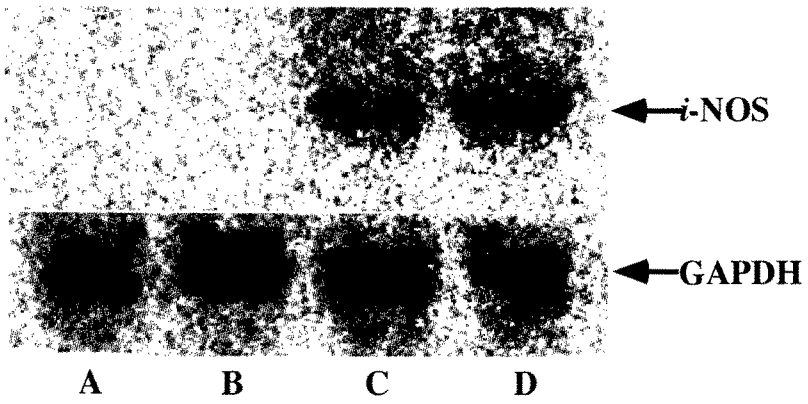

FIGURE 5 Effect of cytokines and/or PQ on iNOS mRNA expression in A549 cells: (A) control, (B) PQ, (C) cytokines, (D) cytokines plus PQ. Eighteen hours after plating in 60mm dishes ($2x10^6$cells/dish), cells were stimulated with the compounds for 9 hrs. This figure is representative of two separate blot experiments performed with 20 μg of total RNA

pressed the LDH release. We studied these findings further by adding a higher concentration of PQ (1 mM) to the medium. The LDH value following incubation with 1 mM PQ was the same as that with 0.2 mM PQ/cytokines. The value doubled with the addition of cytokines. The NOS inhibitor L-NMMA did not prevent LDH release into the medium, whereas ROS scavenging enzymes significantly suppressed the release.

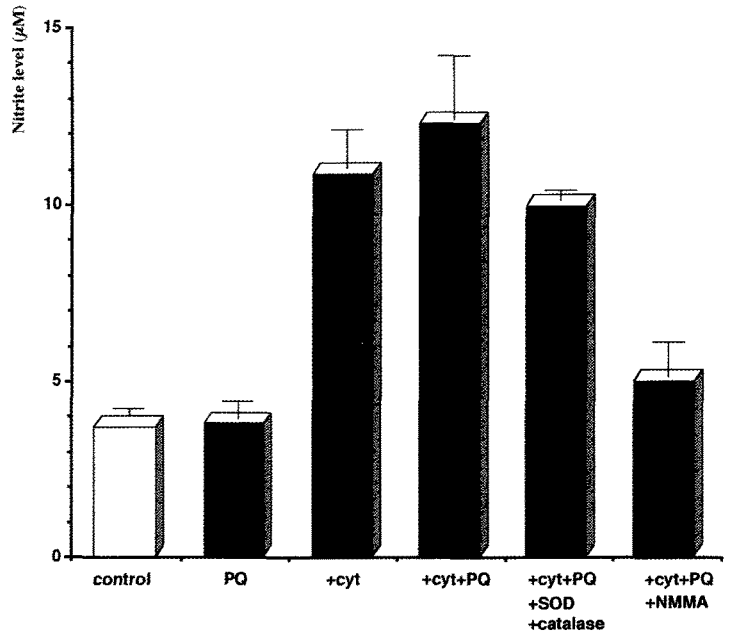

FIGURE 6 Effect of SOD/catalase or L-NMMA on the nitrite accumulation caused by PQ/cytokines. A549 cells plated in 12-well plates (3×10^5/well) were incubated with the additives for 24 hrs. Data are represented as mean ± S.D. (n= 4)

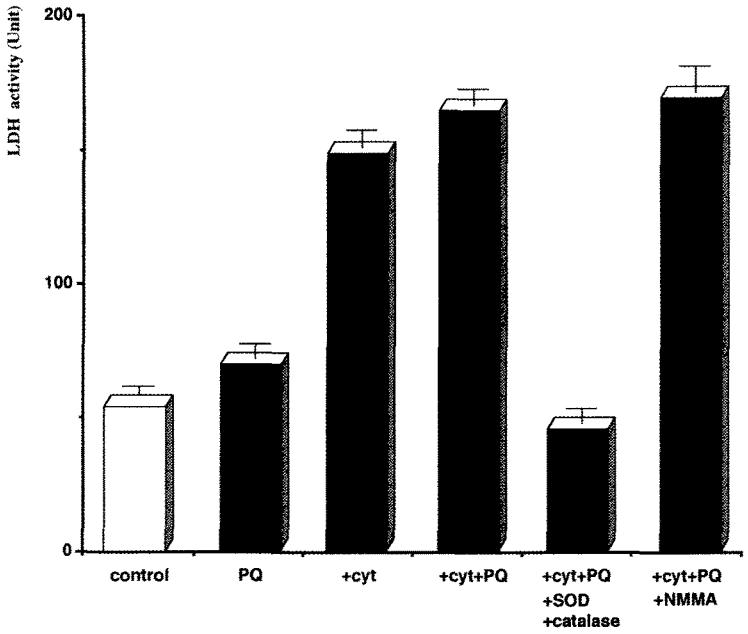

FIGURE 7 Effect of SOD/catalase or L-NMMA on the release of lactate dehydrogenase (LDH) caused by PQ/cytokines. A549 cells plated in 12-well plates (3×10^5/well) were incubated with the additives for 24 hrs. Data are represented as mean ± S.D. (n= 4)

DISCUSSION

The NO donor NOC18 spontaneously releases NO and has a half-life of 21 hrs. We added PQ to the medium at 0.2 mM concentration, which was the minimum toxic dose to membrane, resulting in a slight release of LDH. The NO donor NOC18 had a dangerous effect under the following both conditions; 1) coexistence with PQ and 2) at high concentrations. Although the mechanisms that induced cellular injury under our experimental conditions were not fully understood, they would include generation of other strong oxidants as described elsewhere.[7,8] Other investigators [16] demonstrated that PQ toxicity was not potentiated by the NO donor DETA NONOate, NOC18. In their report, the DETA NONOate alone produced LDH release, and PQ attenuated the release by the NO donor. One of the differences in the experimental conditions is the presence or absence of FBS. As described in elsewhere,[17–19] FBS has relatively high scavenging activity against free radicals. In our experiment, PQ/NOC18 under FBS minus conditions had more dangerous effect than that under FBS plus conditions. Therefore, the discrepancy between our results and their work is probably dependent on the difference in the cells examined. Cappelletti et al.[20] also found differences in the cells examined following PQ-induced actin cytoskeleton disruption. On the other hand, we showed that the cells exposed to low levels of NOC18, 20–50 μM, improved MTT reduction activity. Although we were unable to elucidate how much of the NO from that derived from NOC18 brought about this improvement, this result suggested that a small amount of NO possibly had a protective effect. These data may explain the paradoxical actions of NO described in the literature.[9–11,21,22]

Next, we examined the effect of endogenous NO on PQ toxicity caused by iNOS stimulation with the cytokine mixture. The damage caused by 0.2 mM PQ /cytokoines was completely inhibited by SOD/catalase, but not by NOS inhibitors. The damage caused by higher concentration of PQ (1 mM) / cytokines was also inhibited by SOD/catalase but not by NOS inhibitors. These results showed that ROSs were major players in PQ/cytokine-induced membrane damage and that NO was not a primary stimulant under these in vitro conditions. Recently, Day et al.[16] reported that NOS was a PQ diaphorase resulting in generation of superoxide and a decrease in NO production, and indicated that PQ toxicity involved a loss of NO-related activity. Considering the increase in superoxide, our conclusion does not necessarily seem to be in disagreement with their results. However, nitrite levels were suppressed by adding PQ in their work but not in our experiment. This discrepancy may be due to differences in the cells and cytokine ingredients used. Our results showed a significant increase in LDH release by cytokine mixture alone and further addition of PQ to cytokine mixture showed only a slight increase. Therefore, there was a dangerous effect in cytokine mixture itself in our model system. On the contrary, their experiments showed a small damage by cytokines alone and further addition of PQ gave a significant increase in LDH release. In addition, our activated cells accumulated only 7% of the amount of nitrite that was accumulated by their cytokine (lipopolysaccharide and IFN-γ)-activated macrophages.

PQ exhibits a striking toxicity towards the lung and no effective treatment is available for severe PQ poisoning. The toxicity has generally been attributed to the generation of superoxide by the redox cycling of PQ with cellular diaphorases and oxygen. On the other hand, pro-inflammatory cytokines play a decisive role in inflammatory pathomechanisms, and it is possible that PQ produces pro-inflammatory cytokines in vivo which, in turn, act to induce NO synthesis[23,24] and ROS production.[25] In our experiments, we came to the following conclusions: 1) NO was injurious to the cell membrane when extracellularly high local levels of NO and PQ coexisted. 2) NO, at extracellularly

low levels or at cytokine-induced levels under these experimental conditions, had no deleterious effect. 3) Mitochondrial metabolic activity was possibly improved by a far lower level of NO. These results suggest that the role of NO in PQ-induced cellular damage depends on the amount of NO locally produced in vivo. A large amount of NO may possibly be produced with acute toxicity by the intake of large amounts of herbicide containing PQ.[26] When high levels of NO are produced locally, they will be responsible for local damage.

Acknowledgements

The authors thank Dr. T. Ogura and Dr. S.C. Erzurum for supplying us with the iNOS probe and for technical advice. This study was supported in part by Grants-in-Aid for Scientific Research from the Japanese Ministry of Education, Science and Culture (No. 10670405), and by a Research Project Grant (No.8–506, No.10–604) from Kawasaki Medical School.

References

[1] J.S. Bus, S.D. Aust and J.E. Gibson (1974) Superoxide- and singlet oxygen-catalyzed lipid peroxidation as a possible mechanism of paraquat (methyl viologen) toxicity. *Biochemical and Biophysical Research Communications*, **58**, 749–755.

[2] M.A. Trush, E.G. Mimnaugh, E. Ginsburg and T.E. Gram (1981) In vitro stimulation by paraquat of reactive oxygen-mediated lipid peroxidation on rat lung microsomes. *Toxicology and Applied Pharmacology*, **60**, 279–286.

[3] H.P. Misra and L.D. Gorsky (1981) Paraquat and NADPH-dependent lipid peroxidation in lung microsomes. *Journal of Biological Chemistry*, **256**, 9994–9998.

[4] G.-Y. Wang, K.-I. Hirai and H. Shimada (1992) Mitochondrial breakage induced by the herbicide paraquat in cultured human lung cells. *Journal of Electron Microscopy*, **41**, 181–184.

[5] H.I. Berisha, H. Pakbaz, A. Absood and S.I. Said (1994) Nitric oxide as a mediator of oxidant lung injury due to paraquat. *Proceedings of the National Academy of Sciences USA*, **91**, 7445- 7449.

[6] P. Costantini, V. Petronilli, R. Colonna and P. Bernardi (1995) On the effects of paraquat on isolated mitochondria. Evidence that paraquat causes opening of the cyclosporin A-sensitive permeability transition pore synergistically with nitric oxide. *Toxicology*, **99**, 77–88.

[7] J.S. Beckman, T.W. Beckman, J. Chen, P.A. Marshall and B.A. Freeman (1990) Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proceedings of the National Academy of Sciences USA*, **87**, 1620–1624.

[8] W.H. Koppenol, J.J. Moreno, W.A. Pryor, H. Ischiropoulos and J. S. Beckman (1992) Peroxynitrite, a cloaked oxidant formed by nitric oxide and superoxide. *Chemical Research in Toxicology*, **5**, 834–842.

[9] D.A. Wink, I. Hanbauer, M.C. Krishna, W. DeGraff, J. Gamson and J.B. Mitchell (1993) Nitric oxide protects against cellular damage and cytotoxicity from reactive oxygen species. *Proceedings of the National Academy of Sciences USA*, **90**, 9813–9817.

[10] W.Y. Su, B.J. Day, B.H. Kang, J.D. Crapo, Y.C. Huang and L.Y. Chang (1996) Lung epithelial cell-released nitric oxide protects against PMN-mediated cell injury. *American Journal of Physiology*, **271**, L581-L586.

[11] H.H. Gutierrez, B. Nieves, P. Chumley, A. Rivera and B.A. Freeman (1996) Nitric oxide regulation of superoxide-dependent lung injury: oxidant-protective actions of endogenously produced and exogenously administered nitric oxide. *Free Radical Biology and Medicine*, **21**, 43–52.

[12] C. Köpel, C. von Wissmann, D. Barckow, R. Rossaint, K. Falke, G. Stoltenburg-Didinger and N. Schnoy (1994) Inhaled nitric oxide in advanced paraquat intoxication. *Journal of Toxicology – Clincial Toxicology*, **32**, 205–214.

[13] M.S. Rose, L.L. Smith and I. Wyatt (1974) Evidence for energy-dependent accumulation of paraquat into rat lung. *Nature*, **252**, 314–315.

[14] D.J. Giard, S.A. Aaronson, G.J. Todaro, P. Arnstein, J.H. Kersey, H. Dosik and W.P. Parks (1973) *In vitro* cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. *Journal of the National Cancer Institute*, **51**, 1417–1423.

[15] T. Mosmann (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, **65**, 55–63.

[16] B.J. Day, M. Patel, L. Calavetta, L.Y. Chang and J.S. Stamler (1999) A mechanism of paraquat toxicity involving nitric oxide synthase. *Proceedings of the National Academy of Sciences USA*, **96**, 12760–12765.

[17] A. Baret and I. Emerit (1983) Variation of superoxide dismutase levels in fetal calf serum. *Mutation Research*, **121**, 293–297.

[18] M.E. Holt, M.E. Ryall and A.K. Campbell (1984) Albumin inhibits human polymorphonuclear leucocyte luminol-dependent chemiluminescence: evidence for oxygen radical scavenging. *British Journal of Experimental Pathology*, **65**, 231–241.

[19] M.J. Dumoulin, R. Chahine, R. Atanasiu, R. Nadeau and M.A. Mateescu (1996) Comparative antioxidant and cardioprotective effects of ceruloplasmin, superoxide dismutase and albumin. *Arzneimittelforschung*, **46**, 855–861.

[20] G. Cappelletti, C. Incani and R. Maci (1994) Paraquat induces irreversible actin cytoskeleton disruption in cultured human lung cells. *Cell Biology and Toxicology*, **10**, 255–263.

[21] S. Moncada, R.M. Palmer and E.A. Higgs (1991) Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacological Reviews*, **43**, 109–42.

[22] R. Radi, J.S. Beckman, K.M. Bush and B.A. Freeman (1991) Peroxynitrite-induced membrane lipid peroxidation: the cytotoxic potential of superoxide and nitric oxide. *Archives of Biochemistry and Biophysics*, **288**, 481–487.

[23] P.K. Chatterjee, G.M. Hawksworth and J.S. McLay (1999) Cytokine-stimulated nitric oxide production in the human renal proximal tubule and its modulation by natriuretic peptides: A novel immunomodulatory mechanism? *Experimental Nephrology*, **7**, 438–448.

[24] U. Zumsteg, S. Frigerio and G.A. Hollander (2000) Nitric oxide production and Fas surface expression mediate two independent pathways of cytokine-induced murine beta-cell damage. *Diabetes*, **49**, 39–47.

[25] X.S. Cheng, H. Shimokawa, H. Momii, J. Oyama, N. Fukuyama, K. Egashira, H. Nakazawa and A. Takeshita (1999) Role of superoxide anion in the pathogenesis of cytokine-induced myocardial dysfunction in dogs in vivo. *Cardiovascular Research*, **42**, 651–659.

[26] M. Tomita, T. Okuyama and K. Hidaka (1999) Changes in mRNAs of inducible nitric oxide synthase and interleukin-1ß in the liver, kidney and lung tissues of rats acutely exposed to paraquat. *Legal Medicine*, **1**, 127–134.