Phenanthraquinone Affects Endothelial Nitric Oxide Synthase Activity through Modification of the Thiol Group: An Alternative Inhibition Mechanism

Keiko Taguchi,[a] Yoshito Kumagai,*[,b] Akiko Endo,[a] Makoto Kikushima,[c] Yuji Ishii,[b] and Nobuhiro Shimojo[b]

[a]Master's Program in Environmental Sciences, [b]Department of Environmental Medicine, Institute of Community Medicine, and [c]Doctoral Program in Medical Sciences, University of Tsukuba, 1–1–1 Tennodai, Tsukuba, Ibaraki 305–8575, Japan

(Received July 10, 2001; Accepted July 25, 2001)

Phenanthraquinone (PQ) is a component of diesel exhaust particles which inhibits nitric oxide synthase (NOS) activity by shunting electrons away from the normal catalytic pathway of the enzyme and which can oxidize proximal protein sulfhydryls. In the present study, we examined the possibility that PQ may also modify critical thiol residues on endothelial NOS (eNOS), leading to a disruption of catalytic activity. PQ and the thiol-modifying agent *N*-ethylmaleimide (NEM) suppressed NO formation from L-arginine by the total membrane fraction of bovine aortic endothelial cells in a concentration-dependent manner. The dithiol agent dithiothreitol (DTT) completely blocked NEM-mediated inhibition of eNOS activity. In contrast, PQ-inhibited eNOS activity was reduced by DTT, but not by the monothiol agent glutathione. These results suggest that PQ-mediated suppression of eNOS activity involves not only uncoupling of the electron transport of this enzyme, but also modification of presumably the proximal protein sulfhydryls that play an important role in the maximal catalytic activity.

Key words —— phenanthraquinone, diesel exhaust particle, nitric oxide synthase, thiol

INTRODUCTION

Nitric oxide (NO) is an endogenously generated species with a diverse array of physiological functions.[1] It is biosynthesized from L-arginine by a family of NO synthases (NOSs). Impairment of NO produced by endothelial NOS (eNOS) is implicated in the pathophysiology of vascular diseases.[2,3]

Epidemiologic studies[4–6] and *in vitro* experiments[7] have suggested that the chemical components of diesel exhaust particles in urban air contribute to impairment of vasorelaxation. We reported previously that phenanthraquinone (PQ), a relatively abundant quinone in diesel exhaust particles,[8] interacts with the P450 reductase domain on neuronal NOS (nNOS) and thus inhibits NO formation by uncoupling the electron transport of the enzyme.[9] Subsequently, we reported that PQ was a more potent inhibitor of eNOS than of nNOS but the inhibition characteristics of PQ on eNOS activity were almost the same as on nNOS.[10] Interestingly, it was shown that decreased NO formation in endothelium by PQ was associated with the impairment of NO-dependent vasorelaxation of rat aorta and the elevation of blood pressure.[10] Our data suggest that PQ contributes to the endothelial dysfunction caused by diesel exhaust particles.

Several reports indicated that thiol groups in eNOS are essential for maximal enzyme activity.[11–14] Our recent studies have demonstrated that PQ interacts readily with dithiol groups, but not monothiol groups, thereby oxidizing proximal protein sulfhydryls.[15] Thus we hypothesized that inhibition of eNOS activity by PQ may be due not only to the shunting of electrons away from the normal catalytic pathway, but also modifying critical thiols of the enzyme. In the present study, we examined the involvement of thiol modification in the PQ-mediated inhibition of eNOS activity using bovine aortic endothelial cells (BAEC).

MATERIALS AND METHODS

Materials —— The chemicals and proteins were obtained as follows: PQ, dithiothreitol (DTT), and *N*-ethylmaleimide (NEM) from Nacalai Tesque, Inc. (Kyoto, Japan); L-arginine, thioredoxin (*Spirulina* sp.), and thioredoxin reductase (*Escherichia coli*) from Sigma Chemical Co. (St. Louis, MO, U.S.A.); and L-[2,3-^{3}H]arginine from DuPont/NEN Research Products (Boston, MA, U.S.A.); and cytochrome c from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Calmodulin was purified from bovine brain by the method of Gopalakrishna and Anderson.[16]

*To whom correspondence should be addressed: Department of Environmental Medicine, Institute of Community Medicine, University of Tsukuba, Tsukuba, Ibaraki 305–8575, Japan. Tel.: +81-298-53-3297; Fax: +81-298-53-3039; E-mail: yk-emtu@md.tsukuba.ac.jp

All other chemicals used were obtained from commercial sources and were of the highest grade available.

Determination of eNOS Activity —— BAEC were obtained from Dainippon Pharmaceutical Industrial Co. (Tokyo, Japan) and cultured as described previously.[10] The cells were homogenized in 50 mM Tris-HCl (pH 7.4)-0.1 mM EDTA-0.1 mM EGTA-1 mM phenylmethylsulfonyl fluoride-leupeptin (1 μg/ml). The homogenate was centrifuged at $100000 \times g$ for 60 min to isolate membrane-bound eNOS. The resulting pellets were suspended in homogenate buffer containing 2.5 mM $CaCl_2$ according to the method of Patel and Block.[14] Suspensions obtained were frozen under liquid nitrogen and stored at –70°C before use.

Incubation mixtures (0.1 ml) consisted of a total membrane preparation of BAEC (0.11–0.13 mg of protein), various concentrations of PQ (dissolved in dimethyl sulfoxide), complete medium (20 nM [2,3-^{3}H]arginine, 50 μM L-arginine, 100 μM NADPH, 10 μM 6*R*-5,6,7,8-tetrahydro-L-biopterin, 2 mM $CaCl_2$, 1 μg of calmodulin) and 20 mM HEPES (pH 7.4). After the enzyme solution was preincubated with PQ in the presence and absence of thiol compounds at 37°C for 5 min, reactions were initiated by addition of the complete medium, and incubations were carried out at 37°C for 30 min. NOS activity was determined by monitoring the formation of citrulline.[17] Briefly, each incubation was terminated by the addition of 2 ml of cold stop buffer (20 mM sodium acetate buffer [pH 5.5]-1 mM citrulline-2 mM EDTA-0.2 mM EGTA). A portion (2 ml) of the mixture was applied to a column packed with AG50W-X8 resin (1 ml), which had been extensively equilibrated with the stop buffer, and then the column was washed with 2 ml of water. A sample (1 ml) of the collected eluate was mixed with 5 ml of a scintillation cocktail and the radioactivity was determined by using a Beckman LS-600 scintillation counter. Protein concentration was measured by the method of Bradford[18] with bovine serum albumin as the standard.

Determination of IC_{50} Value —— The eNOS activity in the presence of different concentrations of quinones were analyzed by a nonlinear regression program using PRISM version 3.0 (Graph Pad Software, Inc., San Diego, CA, U.S.A.) to calculate the IC_{50} value.

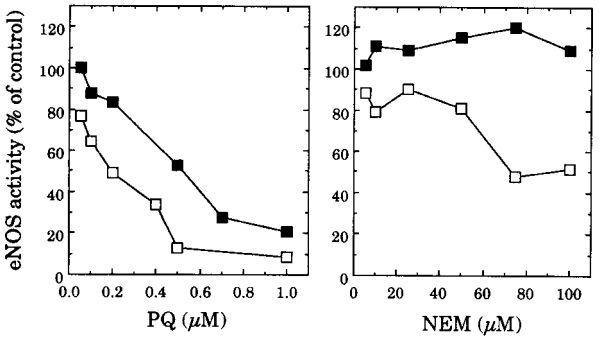

Fig. 1. Effect of DTT on Inhibition of eNOS Activity by the BAEC Preparation by PQ and NEM

Open square, –DTT; closed square, +DTT. The enzyme preparation (112–134 μg of protein) was incubated with different concentrations of PQ or NEM in the presence and absence of DTT (100 μM) at 25°C for 30 min. Then production of NO, determined by citrulline formation from L-arginine, was performed under conditions described in Materials and Methods. The control enzyme activity in BAEC preparation was 22.4 pmol of citrulline formed/mg protein/min. Each point is the mean of two to six determinations.

RESULTS AND DISCUSSION

Basal NO production, determined by citrulline formation, by an isolated membrane fraction of BAEC was unaffected when the dithiol agent DTT (100 μM) was omitted from the incubation mixture. NEM, which reacts readily with sulfhydryl groups, supressed eNOS activity. At NEM 25, 50, and 75 μM, eNOS was inhibited by 10%, 19%, and 52%, respectively. The presence of DTT, however, completely abolished NEM-mediated inhibition (Fig. 1). In contrast, concentration-dependent inhibition of eNOS activity by PQ was reduced by the addition of DTT 100 μM (without DTT, IC_{50} value = 0.22 μM; with DTT, IC_{50} value = 0.56 μM) under the conditions shown in Fig. 1. Such a protective effect of DTT on the PQ-mediated inhibition of eNOS activity was not seen with the monothiol agent glutathione instead of DTT (data not shown). The protective effect of DTT on eNOS activity was abolished at PQ more than 10 μM (data not shown). These results suggest that dithiol, but not monothiol, is essential for partial protection against inhibition of eNOS activity caused by PQ and that the DTT effect appears to be efficient at lower PQ concentration.

We have found that PQ reacts readily with the total membrane fraction of BAEC, resulting in oxidation of protein sulfhydryls.[15] Thus the present results suggest that DTT may compensate for free thiol groups of eNOS in BAEC that are able to react with PQ. However, the inhibition processes of PQ and NEM appeared to be different because modification

Table 1. Effects of Thioredoxin/Thioredoxin Reductase System on Inhibition by PQ of eNOS Activity

Addition	eNOS activity (pmol/min/mg protein)
None (control)	33.9 ± 3.2
+Thioredoxin/thioredoxin reductase	72.4 ± 2.9*
+PQ (0.5 μM)	18.0 ± 1.6*
+Thioredoxin/thioredoxin reductase +PQ (0.5 μM)	15.7 ± 2.3**

BAEC preparation (0.21 mg protein) was preincubated with thioredoxin 0.1 μM, thioredoxin reductase 0.4 μM, and PQ in 20 mM HEPES (pH 7.6) at 37°C for 5 min. Then the reaction was initiated under the conditions described in Materials and Methods. Each value is the mean ± S.D. of three to five determinations. *$p < 0.01$ *vs.* control; **$p < 0.01$ *vs.* + thioredoxin/thioredoxin reductase system.

of the reactive sulfhydryl groups of eNOS by PQ contributed in part to the suppression of eNOS activity, whereas that by NEM was the sole factor in the inhibition of enzyme activity.

Intracellular thiol oxidation (*i.e.*, conversion of thiols to disulfides) can be reversed by the thioredoxin/thioredoxin reductase system in the presence of NADPH.[19] To confirm the importance of thiol groups in the maximal activity of eNOS, we added a thiol-regenerating system to the eNOS enzyme preparation. Basal NO formation by an isolated membrane preparation of BAEC was enhanced by approximately 2.1-fold by the addition of the protein disulfide reductase system thioredoxin/thioredoxin reductase, as shown in Table 1. Significantly, eNOS activity was markedly inhibited (53% of control) by PQ at a concentration of 0.5 μM (Table 1). However, the thiol-regenerating system failed to restore the reduction of eNOS activity caused by PQ (Table 1). This result was somewhat unexpected, since the thioredoxin/thioredoxin reductase system should catalyze conversion of disulfides caused by PQ to active thiols. A reasonable explanation for this discrepancy is that PQ may also affect the proximal thiol groups of not only eNOS but also thioredoxin (or thioredoxin reductase) because both proteins have a vicinal thiol group on the active center.[19]

The results of the present study indicate that the inhibition of eNOS by PQ may have multiple mechanisms. As previously described, PQ may disrupt the electron pathway in NOS, resulting in an inhibition of NO formation.[9,10] As described herein, another mechanism of NOS inhibition exists whereby PQ catalyzes the modification of thiols required to maintain eNOS activity. The critical target thiols may be on eNOS itself or other cellular thiols capable of maintaining eNOS thiols in the reduced state.

Acknowledgements We wish to thank Dr. Jon M. Fukuto, Department of Molecular and Medical Pharmacology, UCLA School of Medicine, for helpful comments. This research was supported in part by Grants-in-Aid #11877398 and #13672340 (YK) for Scientific Research from the Ministry of Education, Science, Culture and Sport of Japan, by the Naito Foundation (YK), and by a fund (University Research Project, YK) from the University of Tsukuba.

REFERENCES

1) Moncada, S., Palmer, R. M. J. and Higgs, E. A. (1991) Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol. Rev.,* **43**, 109–142.
2) Umans, J. G. and Levi, R. (1995) Nitric oxide in the regulation of blood flow and arterial pressure. *Annu. Rev. Physiol.*, **57**, 771–790.
3) Kelly, R. A., Balligand, J. L. and Smith, T. W. (1996) Nitric oxide and cardiac function. *Circ. Res.*, **79**, 363–380.
4) Dockery, D. W., Pope, A. C., III, Xu, X., Spengler, J. D., Ware, J. H., Fay, M. E., Ferris, B. G., Jr. and Speizer, F. E. (1993) An association between air pollution and mortality in six U.S. cities. *N. Engl. J. Med.*, **329**,1753–1759.
5) Pope, C. A., III, Thun, M. J., Namboodiri, M. M., Dockery, D. W., Evans, J. S., Speizer, F. E. and Heath, C. W., Jr. (1995) Particulate air pollution as a predictor of mortality in a prospective study of U.S. adults. *Am. J. Respir. Crit. Care Med.*, **151**, 669–674.
6) Borja-Aburto, V. H., Castillejos, M., Gold, D. R., Bierzwinski, S. and Loomis, D. (1998) Mortality and ambient fine particles in southwest Mexico City, 1993–1995. *Environ. Health Perspect.*, **106**, 849–855.
7) Ikeda, M., Suzuki, M., Watarai, K., Sagai, M. and Tomita, T. (1995) Impairment of endothelium-dependent relaxation by diesel exhaust particles in rat thoracic aorta. *Jpn. J. Pharmacol.*, **68**, 183–189.
8) Schuetzle, D. (1983) Sampling of vehicle emissions for chemical analysis and biological testing. *Environ. Health Perspect.*, **47**, 65–80.
9) Kumagai, Y., Nakajima, H., Midorikawa, K., Homma-Takeda, S. and Shimojo, N. (1998) Inhibition of nitric oxide formation by neuronal nitric oxide synthase by quinones: Nitric oxide synthase as a quinone reductase. *Chem. Res. Toxicol.*, **11**, 608–613.

10) Kumagai, Y., Hayashi, T., Miyauchi, T., Endo, A., Iguchi, A., Kiriya-Sakai, M., Sakai, S., Yuki, K., Kikushima, M. and Shimojo, N. (2001) Phenanthraquinone inhibits eNOS activity and suppresses vasorelaxation. *Am. J. Physiol.*, **281**, R25–R30.
11) Murphy, M. E., Piper, H. M., Watanabe, H. and Sies, H. (1991) Nitric oxide production by cultured aortic endothelial cells in response to thiol depletion and replenishment. *J. Biol. Chem.*, **266**, 19378–19383.
12) Zembowicz, A., Hatchett, R. J., Radziszewsi, W. and Gryglewsi, R. J. (1993) Inhibition of endothelial nitric oxide synthase by ebselen. Prevention by thiol suggests the inactivation by ebselen of a critical thiol essential for the catalytic activity of nitric oxide synthase. *J. Pharmacol. Exp. Ther.*, **267**, 1112–1117.
13) Marczin, N., Ryan, U. S. and Catravas, J. D. (1993) Sulfhydryl-depleting agents, but not deferoxamine, modulated EDRF action in cultured pulmonary arterial cells. *Am. J. Physiol.*, **265**, L220–L227.
14) Patel, J. M. and Block, E. R. (1995) Sulfhydryl-disulfide modification and the role of disulfide oxidoreductases in regulation of the catalytic activity of nitric oxide synthase in pulmonary artery endothelial cells. *Am. J. Respir. Cell Mol. Biol.*, **13**, 352–359.
15) Kumagai, Y., Hayashi, T., Endo, A., Iguchi, A., Yuki, K., Miyauchi, T. and Shimojo, N. (2000) A novel pharmacological action of phenanthraquinone, a component of diesel exhaust particles: inhibition mechanisms of endothelial nitric oxide synthase and suppression of vasorelaxation. *Nitric Oxide*, **4**, p. 238.
16) Gopalakrishna, R. and Anderson, W. B. (1982) Ca^{2+}-induced hydrophobic site on calmodulin: Application for purification of calmodulin by phenyl-Sepharose affinity chromatography. *Biochem. Biophys. Res. Commun.*, **104**, 830–836.
17) Bredt, D. S. and Snyder, S. H. (1990) Isolation of nitric oxide synthase, a calmodulin-requiring enzyme. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 682–685.
18) Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.
19) Holmgren, A. (1985) Thioredoxin. *Annu. Rev. Biochem.*, **54**, 237–271.