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

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

aMaster’s Program in Environmental Sciences, bDepartment of Environmental Medicine, Institute of Community Medicine, and cDoctoral Program in Medical Sciences, University of Tsukuba, 1–1–1 Tennodai, Tsukuba, Ibaraki 305–8575, Japan
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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 studies4–6) and in vitro experiments7) 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-[2,3-3H]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)
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 \(\mu\)g/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 \([2,3\text{-}^3\text{H}]\)arginine, 50 \(\mu\)M L-arginine, 100 \(\mu\)M NADPH, 10 \(\mu\)M 6R-5,6,7,8-tetrahydro-L-biopterin, 2 mM CaCl\(_2\), 1 \(\mu\)g 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\textsubscript{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\textsubscript{50} value.

**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 \(\mu\)M) was omitted from the incubation mixture. NEM, which reacts readily with sulphhydryl groups, suppressed eNOS activity. At NEM 25, 50, and 75 \(\mu\)M, 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 \(\mu\)M (without DTT, IC\textsubscript{50} value = 0.22 \(\mu\)M; with DTT, IC\textsubscript{50} value = 0.56 \(\mu\)M) 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 \(\mu\)M (data not shown). These results suggest that diethiol, 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 sulphhydrils.\(^{13}\) 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

<table>
<thead>
<tr>
<th>Addition</th>
<th>eNOS activity (pmol/min/mg protein)</th>
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<tr>
<td>None (control)</td>
<td>33.9 ± 3.2</td>
</tr>
<tr>
<td>Thioredoxin/thioredoxin reductase</td>
<td>72.4 ± 2.9*</td>
</tr>
<tr>
<td>PQ (0.5 µM)</td>
<td>18.0 ± 1.6*</td>
</tr>
<tr>
<td>Thioredoxin/thioredoxin reductase</td>
<td>15.7 ± 2.3**</td>
</tr>
<tr>
<td>PQ (0.5 µM)</td>
<td></td>
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BAEC preparation (0.21 mg protein) was preincubated with thioredoxin 0.1 µM, thioredoxin reductase 0.4 µM, and PQ in 20 µM 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.

The critical target thiols may be on eNOS itself or other cellular thiols capable of maintaining eNOS thiols in the reduced state.

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