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Inhibition of nitric oxide formation and superoxide generation during reduction of LY83583 by neuronal nitric oxide synthase

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Abstract

6-Anilino-5,8-quinolinedione (LY83583) has been widely used as an agent to reduce levels of nitric oxide (NO)-dependent cGMP in tissues. We report here that suppression of NO formation and production of superoxide during enzymatic reduction of LY83583 by neuronal NO synthase appeared to be potentially involved in the pharmacological action caused by LY83583. LY83583 suppressed neuronal NO synthase activity of $20,000 \times g$ rat cerebellar supernatant preparation in a concentration-dependent manner (IC_{50} value = 12.9 μM). A kinetic study revealed that LY83583 is a competitive inhibitor with respect to NADPH, with a K_i value of 2.57 μM. With purified neuronal NO synthase it was found that LY83583 was a potent inhibitor of NO formation by the enzyme and served as efficient substrate for reduction with a specific activity of 173 nmol of NADPH oxidized per mg of protein per minute. The reductase activity was stimulated about 19.8-fold by addition of $CaCl_2$/calmodulin, indicating that the presence of $CaCl_2$/calmodulin is essential to express maximal activity of LY83583 reduction. Although LY83583 was a good substrate for both NADPH-cytochrome *P*450 reductase (*P*450 reductase) and DT-diaphorase, these flavin enzymes-catalyzed reductions of LY83583 were less than the neuronal NO synthase-mediated reduction in the presence of $CaCl_2$/calmodulin. Enzymatic generation of superoxide during reduction of LY83583 by neuronal NO synthase, *P*450 reductase or DT-diaphorase was confirmed by electron spin resonance (ESR) experiments. Thus the present results indicate that a benzoquinone derivative LY83583 appears to interact with the *P*450 reductase domain on neuronal NO synthase, resulting in inhibition of NO formation and superoxide generation, which is involved in suppression of intracellular cGMP content.

Keywords: LY83583; Nitric oxide (NO) synthase; Superoxide; Quinone; cGMP

1. Introduction

6-Anilino-5,8-quinolinedione (LY83583) lowers intracellular cGMP in various tissues (Schmidt et al., 1985; Diamond and Chu, 1985; MacLeod et al., 1987; Barbier and Lefebvre, 1992) by different mechanisms. Mülsch et al. (1988) had reported that LY83583 inhibits soluble guanylate cyclase which catalyzes the conversion of GTP to cGMP and is activated by nitric oxide (NO) (Dierks and Burstyn, 1996). It was also shown that this compound suppresses not only endothelium-dependent vasorelaxation (MacLeod et al., 1987; Mülsch et al., 1988; Cherry et al., 1990; Støen et al., 1997), an effect which is mediated by enzymatically formed NO, but also endothelium-independent relaxation induced by exogenous NO generated from nitrovasodilator drugs (Mülsch et al., 1988; Furchgott and Jothianandan, 1991; Barbier and Lefebvre, 1992; Kontos and Enoch, 1993). Although possible mechanisms to explain the LY83583-promoted pharmacological action have been suggested by others (Mülsch et al., 1988; Gidari et al., 1989; Kontos and Enoch, 1993; Sundqvist and Axelsson, 1993; Luo et al., 1995), details of the mechanism for inhibition of NO formation by NO synthase and superoxide generation by LY83583 still remain obscure.

Endogenous formation of NO from L-arginine is catalyzed by NO synthase. Isozymes of NO synthase consisting of neuronal-, endothelial- and inducible forms have in

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common a NADPH-cytochrome *P*450 reductase (*P*450 reductase) domain (Lowenstein and Snyder, 1992), which is capable of transferring electrons from NADPH to artificial acceptors (Klatt et al., 1992). We have recently found that quinoid compounds, which undergo one- or two-electron reduction by flavoenzymes such as *P*450 reductase and DT-diaphorase (Chesis et al., 1984; O'Brien, 1991), are potent inhibitors of rat neuronal NO synthase activity and that the enzyme can serve as a quinone reductase which shunts electrons away from oxygen activation and L-arginine oxidation (Kumagai et al., 1998). These findings suggested that neuronal NO synthase would effectively reduce quinones, causing a marked decrease in the formation of NO from L-arginine. Since LY83583 possesses a benzoquinone structure, it was assumed that the blockage of electron flow from NADPH to suppress NO formation during reduction of this quinone by NO synthase, could possibly be associated with a decrease in cGMP content. Thus the present study was designed to test whether or not neuronal NO synthase is capable of reducing LY83583, and in the process, lead to superoxide generation in the presence of NADPH. We also examined the contribution of other flavoproteins, *P*450 reductase and DT-diaphorase, to the generation of active oxygen species from LY83583.

2. Materials and methods

2.1. Materials

LY83583 was obtained from Calbiochem–Novabiochem (La Jolla, CA, USA). L-Arginine and L-[2,3-^{3}H]arginine were purchased from Sigma (St. Louis, MO, USA) and DuPont/NEN Research Products (Boston, MA, USA), respectively. DEAE-Sephacel, 2′5′-ADP Sepharose 4B and calmodulin Sepharose 4B were obtained from Pharmacia LKB Biotechnology (Uppsala, Sweden). 5,5′-Dimethyl-1-pyrroline *N*-oxide (DMPO) was purchased from Labotech (Tokyo, Japan). DT-diaphorase was obtained from TOYOBO (Osaka, Japan). All other chemicals used were of the highest grade available.

2.2. Enzyme preparation

Wistar male rats (5 weeks) were decapitated and the cerebellums were homogenized with 2 vol of 50 mM Tris–HCl (pH 7.4)–0.1 mM EDTA–0.1 mM EGTA–0.5 mM dithiothreitol–1 μM pepstatin–2 μM leupeptin–1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 20,000 × *g* for 60 min. Supernatants obtained were frozen under liquid nitrogen and kept at −70°C until use. Neuronal NO synthase was purified from the 20,000 × *g* supernatant preparation of rat cerebellum by DEAE-Sephacel column chromatography, 2′5′-ADP Sepharose 4B column chromatography, and calmodulin Sepharose 4B column chromatography according to the methods of Bredt and Snyder (1990) and Schmidt et al. (1991). *P*450 reductase was purified from liver microsomes from phenobarbital-treated Sprague–Dawley rats by the method of Kumagai et al. (1994a). Calmodulin was purified from bovine brain by the method of Gopalakrishna and Anderson (1982). Cu^{2+},Zn^{2+}-superoxide dismutase was purified from bovine erythrocytes as described previously (Kumagai et al., 1994b). Each final preparation of purified protein showed a single bond on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein concentration was measured by the method of Bradford (1976) with bovine serum albumin as a standard.

2.3. No formation

Incubation mixtures (0.1 ml) contained enzyme preparation, various concentrations of LY83583, 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 40 mM HEPES buffer (pH 7.6). Reactions were initiated by addition of the complete medium and were carried out at 37°C for 10 min. LY83583 was dissolved in dimethyl sulfoxide (DMSO) and the maximal volume of DMSO was kept to 20 μl/ml of assay mixture. Under these conditions, NO synthase activity was slightly affected by DMSO (10.7% inhibition of control). After the reaction, production of citrulline from arginine was performed according to the method of Bredt and Snyder (1990).

2.4. Reduction of LY83583

The reductase activity of LY83583 was determined by NADPH oxidation at 340 nm using an extinction coefficient of 6.22 mM^{-1} cm^{-1}, with a Shimadzu UV-1600 double beam spectrophotometer (Kyoto, Japan). Briefly, incubation mixtures (1.2 ml) contained purified neuronal NO synthase, *P*450 reductase or DT-diaphorase (added to the sample cuvette only), 10–100 μM LY83583, complete medium described above and 40 mM HEPES buffer (pH 7.6). Reactions were initiated by addition of the complete medium to the reference and sample cuvettes. The LY83583-dependent NADPH oxidation was estimated by subtracting the enzymatic NADPH consumption in the presence of DMSO only.

2.5. Detection of superoxide

Superoxide generated during reduction of LY83583 by flavin enzymes was identified as DMPO-OOH adduct. Electron spin resonance (ESR) was performed at 25°C using a JES-TE200 spectrometer (JEOL, Tokyo, Japan) as reported previously (Kumagai et al., 1997). Incubation mixture (0.2 ml) containing 0.1 mM LY83583, various amounts of enzymes, 0.1 mM NADPH, 100 mM DMPO

and 40 mM Hepes buffer (pH 7.6) was incubated at 25°C for 2 min unless otherwise noted. The spectrometer settings are indicated in the figure legend.

3. Results

3.1. Inhibition of neuronal NO synthase activity by LY83583

Fig. 1 shows the effect of LY83583 on neuronal NO synthase activity of 20,000 × *g* supernatant preparation of rat cerebellum. LY83583 was a potent inhibitors of NO production as determined by citrulline formation with an IC_{50} value of 12.90 μM which is comparable to that reported by Luo et al. (1995). Addition of GSH (1 mM), Cu^{2+},Zn^{2+}-superoxide dismutase (10 units), catalase (10 units), mannitol (100 mM) or thiourea (10 mM) to the incubation mixture of the neuronal NO synthase preparation in the presence of LY83583 failed to appreciable restore the decrease in NO formation caused by LY83583 (data not shown), suggesting that either covalent modification of the NO synthase protein or generation of potentially destructive active oxygen species was not involved in the LY83583-mediated suppression of neuronal NO synthase activity. As shown in Fig. 2, LY83583 was found to be a competitive inhibitor with respect to NADPH; the K_i value obtained from Dixon plot analysis was 2.57 μM.

3.2. Reduction of LY83583 by neuronal NO synthase and other flavin enzymes

Since neuronal NO synthase is capable not only to form NO from L-arginine but also to reduce a variety of quinones (Kumagai et al., 1998), the possibility of enzyme-catalyzed reduction of LY83583 was tested. Table 1 summarizes

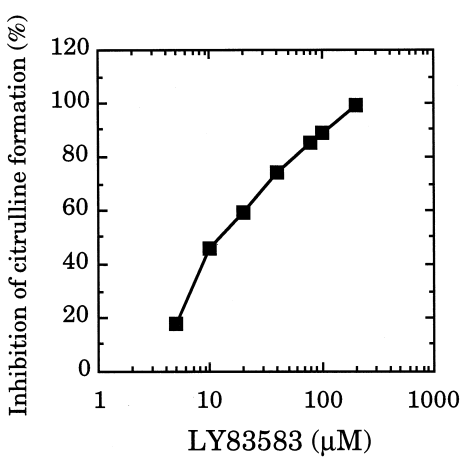

Fig. 1. Inhibition by LY83583 of neuronal NO synthase activity by 20,000 × *g* supernatant preparation of rat cerebellum. Neuronal NO synthase activity was determined as citrulline formed from L-arginine. Each pint is the average of two determinations.

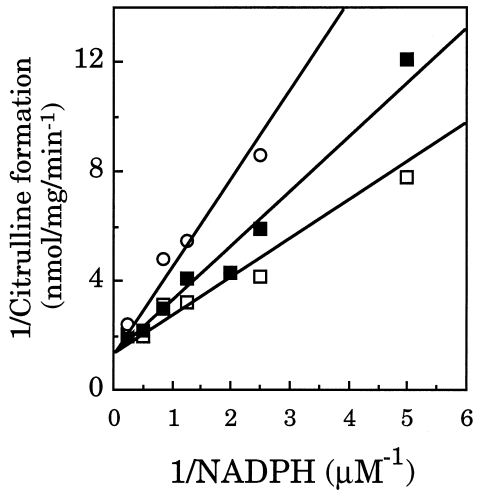

Fig. 2. Lineweaver–Burk plot of NO formation by 20,000 × *g* supernatant preparation of rat cerebellum in the absence and presence of LY83583. NO formation was determined as citrulline formation. Each point is the average of two determinations.

results of reductase activities for LY83583 of flavoenzymes, neuronal NO synthase, *P*450 reductase and DT-diaphorase. In the absence of LY83583 and L-arginine, the rates of neuronal NO synthase-catalyzed NADPH oxidation with and without $CaCl_2$/calmodulin were 981 and 66 nmol/mg/min, respectively. As expected, LY83583 was reduced by neuronal NO synthase (specific activity: 173 nmol of NADPH oxidized/mg/min). While neuronal NO synthase catalyzed NO formation with a specific activity of 647.70 nmol of citrulline formed/mg/min (in the presence of $CaCl_2$/calmodulin), the reduction of LY83583 was stimulated about 19.8-fold by addition of $CaCl_2$/

Table 1
Reduction of LY83583 by neuronal NO synthase, *P*450 reductase and DT-diaphorase

Conditions	Reductase activity of LY83583[a]		
	Neuronal NO synthase	*P*450 reductase	DT-diaphorase
nmol/mg/min (% of control)			
Complete	173 (100)	831 (100)	1716 (100)
+NADH[b] (0.1 mM)	0 (0)	0 (0)	1822 (106)
+$CaCl_2$ /calmodulin	3419 (1976)	479 (58)	1205 (70)
+diphenylene -iodonium (10 μM)	313[c] (181)	638 (77)	2015 (114)

[a] LY83583-dependent NADPH oxidation was estimated by subtracting the enzymatic NADPH consumption in the presence of DMSO only (see Section 2.4).
[b] This was used instead of NADPH (0.1 mM).
[c] Reaction was carried out in the presence of 2 mM $CaCl_2$ and calmodulin (1 μg). Specific activities of NADPH oxidation in the absence of LY83583 (DMSO only) by neuronal NO synthase were 981 nmol/mg/min (with $CaCl_2$ /calmodulin) and 66 nmol/mg/min (without $CaCl_2$ /calmodulin). Each pint is the average of duplicate determinations.

calmodulin, indicating that $CaCl_2$/calmodulin is required for the maximal activity, not only of NO formation (Bredt and Snyder, 1990) but also of reduction of LY83583, like 9,10-phenanthraquinone (Kumagai et al., 1998). Under these conditions, NO formation from L-arginine by neuronal NO synthase was suppressed by LY83583 (IC_{50} value = 29 μM). Although *P*450 reductase and DT-diaphorase reduced LY83583 effectively, the reductase activity of these flavin enzymes was 24% (*P*450 reductase) and 50% (DT-diaphorase) of that of neuronal NO synthase in the presence of $CaCl_2$/calmodulin. In contrast, the presence of $CaCl_2$/calmodulin suppressed either *P*450 reductase- or DT-diaphorase-mediated reduction of LY83583. When NADH was used instead of NADPH, no reductase activity of neuronal NO synthase and *P*450 reductase was measurable whereas reduction of LY83583 by DT-diaphorase was required either pyridine nucleotide as the cofactor. Diphenyleneiodonium (10 μM), which competes with a nucleotide cofactor for binding to NO synthase (Stuehr et al., 1991), blocked the neuronal NO synthase-catalyzed reduction of LY83583 markedly. However, this compound had a negligible effect on the reduction of LY83583 by *P*450 reductase and DT-diaphorase.

3.3. Superoxide generation

If semiquinones, which easily undergo autooxidation to produce superoxide (O'Brien, 1991; Monks et al., 1992), are formed from one-electron reduction of LY83583 by neuronal NO synthase in the presence of NADPH, any active oxygen species generated should be detectable. As shown in Fig. 4, the ESR spectrum was monitored in which the primary species was characteristic of the DMPO-OOH adduct, due to superoxide generation (Fig. 3A), but L-arginine (0.1 mM), a natural substrate for NO synthase, blocked the spin trapping of superoxide (Fig. 3B), which was consistent with the report by Pou et al. (1992). Addition of LY83583 (0.1 mM) to the incubation mixture of neuronal NO synthase resulted in a dominant formation of DMPO-OOH adduct compared to the control incubation in the absence of L-arginine (Fig. 3C), which was completely inhibited by Cu^{2+},Zn^{2+}-superoxide dismutase (Fig. 3D). Similar results, i.e., that superoxide was

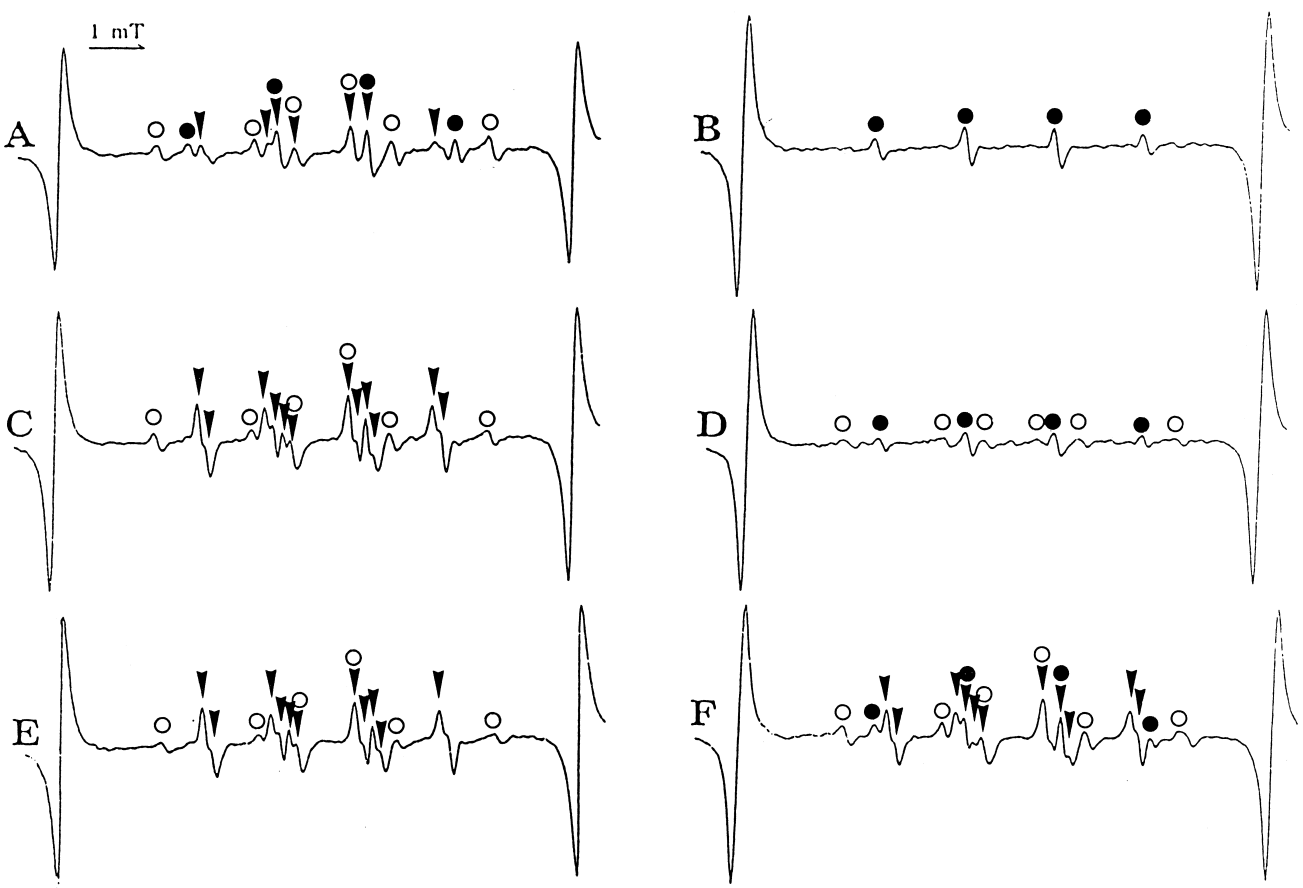

Fig. 3. Generation of active oxygen species during interaction of neuronal NO synthase with NADPH. (A) complete system (0.2 ml) consisted of 40 mM Hepes (pH 7.6), neuronal NO synthase (0.1 μg), BSA (2 mg/ml), 2 μl of DMSO, calmodulin (1 μg), 2 mM $CaCl_2$, 10 μM (6*R*)-5,6,7,8-tetrahydro-L-biopterin, 100 mM DMPO and 0.1 mM NADPH; (B) complete system +0.1 mM arginine; (C) complete system +0.1 mM LY83583; (D) complete system +0.1 mM LY83583 + Cu^{2+},Zn^{2+}-superoxide dismutase (100 units); (E) incubation mixture of *P*450 reductase (1.2 μg); (F) incubation mixture of DT-diaphorase (20 pg). Arrowhead, DMPO-OOH adduct; ●, DMPO-OH adduct; ○, DMPO-methyl adduct. The instrument settings were as follows: modulation width, 100 kHz–0.079 mT; sweep time, 8 min; time constant, 1 s; microwave power, 16 mW; microwave frequency, 9.4251 GHz; magnetic field, 336.5 ± 5 mT. DMPO-OOH adduct: $a_{H\gamma}$, 0.14 mT; $a_{H\beta}$, 1.13 mT; a_N, 1.41mT. DMPO-OH adduct: a_N, 1.49 mT; a_H,, 1.49 mT.

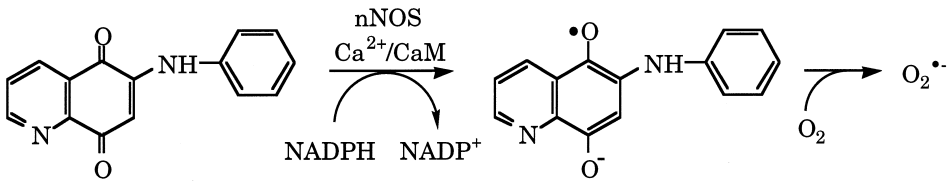

Fig. 4. Neuronal NO synthase-catalyzed reduction of LY83583 and subsequent generation of superoxide. nNOS, neuronal NO synthase; CaM, calmodulin. Theoretically, it is presumed that, during one-electron reduction of 2 mol of quinone by flavin enzyme in the presence of 1 mol of NADPH, 2 mol of semiquinone radical produced reacts with 2 mol of molecular oxygen to form 2 mol of superoxide (Iyanagi, 1987).

generated during interaction of neuronal NO synthase with LY83583, were also observed with *P*450 reductase (Fig. 3E) or DT-diaphorase as enzyme source (Fig. 3F). These DMPO-OOH adducts detected were eliminated by addition of Cu^{2+},Zn^{2+}-superoxide dismutase in both cases (data not shown). In contrast, neither GSH nor dithiothreitol was capable of reducing LY83583, as no DMPO-OOH adduct formation was detected during reaction of this quinoid compound with the thiol agents at a physiological concentration (5 mM).

4. Discussion

LY83583 has been found to reduce the intracellular cGMP concentration by inhibiting the endothelium-derived relaxing factor (Furchgott, 1984) identified as NO, or by generating superoxide, which can inactivate NO (Palmer et al., 1987; Ignarro, 1989). The present results with purified neuronal NO synthase indicate that a decrease in NO formation and generation of superoxide caused by LY83583 result from enzymatic reduction of this quinoid compound by the enzyme. The inhibition of neuronal NO synthase by LY83583 was non-competitive with respect to L-arginine as reported by Luo et al. (1995) but competitive with respect to NADPH (Fig. 2), indicating that LY83583 does not bind to the substrate binding site of neuronal NO synthase but rather binds to or near the NADPH binding site. Our recent studies have shown that quinoid compounds such as 9,10-phenanthraquinone, 1,4-naphthoquinone and menadione, which are reduced by neuronal NO synthase, inhibit NO formation whereas 1,4-benzoquinone, 9,10-anthraquinone, mitomycin C, which have a negligible inhibitory action on neuronal NO synthase activity, were poor substrates for reduction by the enzyme. (Kumagai et al., 1998). Thus it seems likely that LY83583 also binds to the *P*450 reductase domain close to the C-terminal of neuronal NO synthase, thereby shunting the electron flow from the cofactor, resulting in the inhibition of NO formation.

Mülsch et al. (1988) reported previously that LY83583 may require some interaction with intact cells before it can exert its inhibitory action on NO production or NO-mediated smooth muscle relaxation. Gidari et al. (1989), and Barbier and Lefebvre (1992) have proposed that the activity of LY83583 was attributable to its ability to generate superoxide in tissues because Cu^{2+},Zn^{2+}-superoxide dismutase attenuated this compound-mediated inhibition of relaxation, but the exact mechanism for superoxide generation from LY83583 is not known. The chemical contribution to LY83583-promoted superoxide production was almost negligible compared to neuronal NO synthase-dependent reaction. This was suggested by the results of ESR studies, indicating that neuronal NO synthase appears to be a catalyst for one-electron reduction of LY83583 to a semiquinone radical, which reacts readily with molecular oxygen to form superoxide (Fig. 4) (O'Brien, 1991; Monks et al., 1992). Thus these observations point to an alternative mechanism involved in the suppression of intracellular cGMP content by LY83583 and the mechanism is that, like 9,10-phenanthraquinone (Kumagai et al., 1998), LY83583 can act as artificial electron acceptor and thus inhibit NO synthesis and produce superoxide.

Furthermore, enzymatic production of superoxide, accompanied by interaction of LY83583 with *P*450 reductase or DT-diaphorase, was observed (Fig. 3). Immunoblot analysis with antibodies against rat liver *P*450 reductase and DT-diaphorase showed that both enzymes exist in a blood vessel (Kumagai et al., unpublished observation). Peroxynitrite, produced by reaction of superoxide generated with NO (Beckman et al., 1990; Huie and Padmaja, 1993) is thought to fail to activate the guanylate cyclase responsible for producing cGMP. Hence, we suggest that *P*450 reductase and DT-diaphorase also may play an important role in the inactivation of endogenous and exogenous NO by LY83583 after it is reduced.

In conclusion, antineoplastic anthracyclic quinones such as doxorubicin and aclarubicin have been reported to affect NO synthase activity (Luo and Vincent, 1994) and to inhibit endothelium-dependent relaxation of aorta (Wakabayashi et al., 1991). This together with our present results suggested strongly that the pharmacological actions of quinones could be associated with their resulting reduction by NO synthase isozymes. To confirm this hypothesis, an extensive examination of the structure-activity relationships of quinoid compounds, showing inhibition of NO-dependent relaxation and reduction of cGMP content, is now in progress in our laboratory.

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